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für Hals-, Nasen- und Ohrenheilkunde  
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des Fachbereichs Medizin der Philipps-Universität Marburg  
in Zusammenarbeit mit dem Universitätsklinikum Gießen und Marburg GmbH  
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**Cyfra 21-1 as a Serum Tumor Marker for Follow-up of  
Patients with Laryngeal and Hypopharyngeal Squamous Cell  
Carcinoma**

-  
**Inaugural-Disseration**

zur

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Für meine Familie

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## 1. Introduction

Head and neck cancer is a broad definition that covers a range of tumors which arise from the epithelial lining of a number of sites in the upper aerodigestive tract. The most common sites of disease are the oral cavity, pharynx, larynx and nasopharynx. About 90% of the lesions are squamous cell carcinomas. Globally, cancers of the head and neck account for over 5% of malignancies; with more than 500,000 new cases worldwide and over 300,000 attributable deaths recorded in 2002 [1].

Head and neck squamous cell carcinoma are rapidly proliferating tumors. Following the treatment of early stage disease, the most frequent disease related event is the development of a second primary tumor. In advanced disease, local or distant recurrence is common and represented the most common cause of death (45%), followed by comorbidity (21%), treatment related complications (15%) and second primary tumors (9%) [2].

Despite improvement in diagnosis and management, the long term survival rates are among the lowest compared with the major cancers, although for the last 30 years they have almost stay constant [3]. Therefore, effective treatment planning would be enhanced by identification of new prognostic indicators that more accurately reflect the biological behavior of a particular tumor in relation to its host [4]. In this respect, extensive investigation of the prognostic importance of a variety of immunological and histological characteristics of head and neck squamous cell carcinoma has been done in an attempt to identify those features associated with aggressive biological behavior [5].

Generally, distant metastases of carcinomas of the upper aero-digestive tract present with non-specific clinical symptoms, so the detection of distant metastases is difficult. Finding a tumor marker for prediction of impending appearance of distant metastases would lead to better utilization of clinical staging procedures like CT scans, ultrasound, etc. Early detection of tumor progression provides more options for therapy and survival [6].

Serum tumor markers have been accepted as valuable tools for prognosis, and treatment monitoring over the last two decades. Various serum tumor markers such as squamous cell carcinoma antigen (SCCAg), Carcinoembryonic antigen (CEA),

lipid associated sialic acid, SCC marker (TA-4), serum intercellular adhesion molecule-1(S-ICAM-1) etc. have been examined for their value in detecting head and neck cancers. However, due to their low sensitivity, these markers are not clinically useful in HNSCC [6, 7].

Cytokeratins (CK), belonging to the intermediate filament (IF) family of proteins are particularly useful tools for diagnosis in oncology. At present, at least 37 different human CK have been identified of which CK 8, 18, and 19 are the most abundant in simple epithelial cells [8].

CK are subgroubed into type I (40-56.5 kDa) and type II (53-67kDa) CK. Type I are acidic while type II are basic CK, depending on their tissue expression pattern, they have been grouped into simple epithelia specific CK (CK7, 8, 18, 19, 20) and stratified epithelia specific CK (CK4, 5, 13, 14, etc.) [8].

It has been observed that when malignant cells disintegrate, partially degraded CK fragments are released in circulation and can be quantified using various commercially available specific serological assays. The levels of serum markers reflect the tumor burden and are not sensitive enough to be used for screening and early diagnosis of primary cancer. By contrast, the role of serum tumor markers is established in the diagnosis of recurrent disease and in the evaluation of response to treatment [9].

The three most frequently used CK which are being evaluated as serum markers for their utility in clinical applications are tissue polypeptide antigen (TPA), tissue polypeptide specific antigen (TPS), and Cytokeratine fragments 21-1 (Cyfra 21-1). Assays for TPA measure CK 8, 18, and 19 and assays for TPS and Cyfra 21-1 are more specific and measure CK 18 and CK 19 levels, respectively [10].

Cytokeratins are intermediate filaments expressed by all epithelial cells and which appear to be useful markers of epithelial differentiation. Cyfra 21-1 measures cytokeratin fragments of cytokeratin 19 with the aid of two specific monoclonal antibodies (mAbs): BM 19.21 as the capture mAb and KS 19.1 as the detector mAb. The target sites for the Cyfra 21-1 mAbs lie within amino acids 346–367 for BM 19.21 and within amino acids 311–335 for KS 19.1. Cytokeratin 19 consists of 400 amino acids; thus both epitopes are located in the C-terminal helical region of the molecule.

Serum Cyfra 21-1 has been used as a tumor marker for the diagnosis of malignancies of different origin [9].

Cytokeratin fraction 21-1 (Cyfra 21-1) is a well accepted tumor marker with high sensitivity and specificity in non-small-cell lung cancer, especially squamous cell carcinoma (independent prognostic factor) [11]. In SCCHN, the clinical value of Cyfra 21-1 as a tumor marker has been debated inconclusively, probably due to difficulties in finding the appropriate cut-off level [12].

Cyfra 21-1 serum levels in patients with head and neck cancer are generally lower than in patients with lung cancer and they are often even equivalent to levels which are considered normal in lung cancer patients. Cytokeratins are not organ specific, and they appear in all epithelial tumors, as well as in normal epithelium. This is a limitation on the tumor marker potential of Cyfra 21-1 [13, 14].

The aim of this study was to evaluate the importance of Cyfra 21-1 at the time of initial diagnosis and its potential as a tumor marker for follow up of patients with squamous cell carcinoma in two major sub-sites of the head and neck (laryngeal and hypopharyngeal tumors), without determination of a certain cut-off level. Instead, repeated testing Cyfra 21-1 during management and to compare Cyfra 21-1 levels at the time of initial diagnosis with subsequent levels (post-therapy, follow-up) to detect abrupt rise in the serum levels.

## 2. Questions

The following questions require decisive statements:

1. At the time of first diagnosis, is there a correlation between the size of the primary tumor and the Cyfra 21-1 concentration?
2. At the time of first diagnosis, is there a correlation between the extent of lymphogenic metastasis and the Cyfra 21-1 concentration?
3. At the time of first diagnosis, is there a correlation between the histological tumor grading and the Cyfra 21-1 concentration?
4. Is it possible to give statements on the specificity and sensitivity of Cyfra 21-1 as follow-up marker of squamous cell carcinoma of the larynx and hypopharynx - especially with regard to the question of local recurrence and pulmonary metastasis?



### **3. Patients and Methods**

#### **3.1 Patients**

A total of 50 patients with primary diagnosis of laryngeal and hypopharyngeal SCC between 2003 and 2007 in the Dept. of Otolaryngology, Head and Neck Surgery, University of Marburg, Germany, were included in this retrospective evaluation. The diagnosis was confirmed by histological biopsy findings. Tumor extent, nodal involvement, and distant metastases were assessed by a detailed physical examination, endoscopic examination and imaging investigations (B-mode ultrasonography of the neck, chest CT, neck CT, bone scan, liver scan, etc). All patients were staged according to the International Union against Cancer (UICC), TNM classification system [American Joint Committee on Cancer (AJCC), Staging Manual, 6<sup>th</sup> ed. (2002)].

42 patients were men and 8, women. The patients ranged in age from 40 to 82 years. The anatomical sites of HNSCC were, the larynx (n=26), hypopharynx (n=24). Five patients had stage I disease, 8, stage II; 8, stage III; and 29, stage IV disease, according to the International Union against Cancer (UICC) staging system.

The patients were followed periodically in the oncology clinic of the Dept. of Otolaryngology, Head and Neck Surgery, University of Marburg, Germany, with an attempt for the early detection of recurrences, metastases and secondary carcinomas; additionally, they involve adequate pain control therapy. The monthly follow-up of these patients include clinical examinations, routine hematological tests. Computerized tomography and ultrasonography of the neck, chest, and abdomen were performed at three months intervals during the first year, endoscopy was performed once a year but patients with any positive sign during the periodic follow-up examination underwent complete evaluation and investigations including endoscopy and chest CT-scan and if needed restaging procedures.

According to the clinical course of the patients during the period of follow-up, we divide them into two major groups: First group (n=32), all patients in this group had complete remission during one year follow-up, according to the line of management

planned for each of them. Second group (n=18), those patients with local residual disease, recurrence and/ or distant metastases during the follow-up period and all patients with local residual disease or recurrence were confirmed with biopsy and histopathological examination which revealed SCC.

The data for clinical follow-up were available from the medical file for each patient, and Cyfra 21-1 levels were correlated to the clinical course of the patients and this study was independent of any reference values, for example, healthy individuals, because only the change over time of the Cyfra 21-1 serum level in the individual patient was correlated with the individual clinical course.

### **3.2 Methods**

Cyfra 21-1 serum level of 50 patients with laryngeal and hypopharyngeal SCC were evaluated by ECLIA assay [CK-19 Two MAbs Ks 19.1 (aa 311-335) and BM 19.21 (aa 346-367) located within helix 2B of the rod domain (Boehringer, Mannheim, Germany)]. The cytokeratine 19 fragments were detected by the monoclonal antibodies Ks 19-1 and BM 19-21, the antibodies are specific for two different epitopes of cytokeratine 19. The calculated concentration of Cyfra 21-1 was expressed in ng/ml, and the cut-off level of 3.3ng/ml was used according to manufacturer instructions. ECLIA (electrochemiluminescent) is a new method for the determination of cytokeratin 19 (Cyfra 21-1) in the Elecsys 2010 immunoassay system.

The Elecsys<sup>®</sup> 2010 analyser [Boehringer Mannheim (BM)] is based on the ability of the electrochemiluminescent label molecule, a tris (2,2'-bipyridyl) ruthenium (II) complex, to be repeatedly excited by tripropylamine, thus leading to an amplification of light signal that allows the high speed and dynamics of signal generation and measurement. It provides the first test result in 18 min and has a maximum throughput of 86 tests per hour. The system can develop both competitive and sandwich-format electrochemiluminescent assays.

The Elecsys 2010 system is a fully automated immunoassay analyser that can work in batch, random, or stat modes. The automated process consists of the aspiration of

the sample, reagent and microparticles, a first incubation at 37°C, additional reagent pipetting, a second incubation at 37°C, reaction mixture aspiration, and measurement. The analyser also includes a workstation for system programming and can be interfaced to various laboratory computers.

### **Elecsys 2010 Cyfra 21-1 assay**

No preanalytical preparation of reagents is required for the Elecsys 2010 Cyfra 21-1 assay (cat. no. 1820966). In a first incubation of 9 min, 20 µL of sample, a biotinylated monoclonal cytokeratin 19-specific antibody, and a monoclonal cytokeratin 19-specific antibody labeled with a ruthenium complex [a tris (2,2'-bipyridyl) ruthenium (II) complex] react to form a sandwich complex. After the addition of streptavidin-coated microparticles, there is a second incubation for 9 min, and the complex becomes bound to the solid phase via the interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with a phosphate-triethylamine buffer (pH 6.8; Procell®, BM). Application of a voltage to the electrode then induces chemiluminescent emission, which is measured by a photomultiplier.

Calibration for the ECLIA Cyfra 21-1 assay was done using two calibrators (Elecsys 2010) at two different concentrations, 5 and 50 µg/L of analyte. Every instrument-specific calibration curve is generated by a two-point calibration and a master curve provided via the reagent barcode.

Venous blood samples (6 ml) were collected after informed consent was obtained from patients admitted to the Dept. of Otolaryngology, Head and Neck Surgery, University of Marburg, Germany, for treatment of their laryngeal or hypopharyngeal SCC. The samples were allowed to clot, centrifuged at room temperature, and stored at -80 °C until processing.

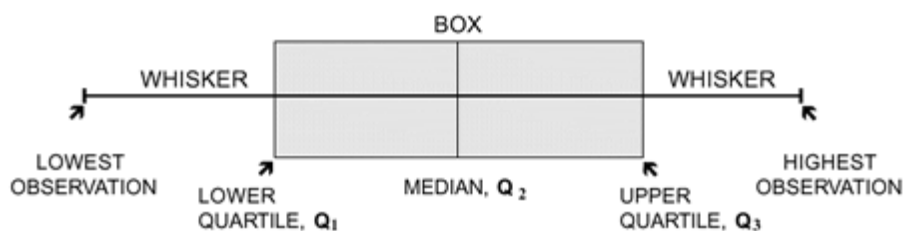
The serum levels of Cyfra 21-1 were collected for each individual patient at the time of primary diagnosis, 6-8 weeks post therapy (either surgery or chemo-radiotherapy or combined), and at least one reading on follow-up in a period extending from 6

months - 1.5 year (available data), and the serum levels of cyfra 21-1 obtained for each individual patient drawn graphically in relation to time of collection as it is more informative and easy to pick up the changes in serum levels at an early stage.

### 3.3 Statistical Analysis

Statistical analysis was performed using **SPSS 15.0 for windows**, a nonparametric tests (**Kruskal-wallis H and Jonckheere-Terpstra Tests**) were used for the type of significance estimate and these tests not assuming the normal distribution or equal group variances. In SPSS, these tests require that the Exact Tests add-on module be installed. A p value of less than 0.05 was considered statistically significant.

(**Boxplots or box and Whisker plot**) also used using SPSS 15.0 for windows. In descriptive statistics, a **boxplot** (also known as a **box-and-whisker diagram** or **plot**) is a convenient way of graphically depicting groups of numerical data through their five-number summaries (the smallest observation, lower quartile (Q1), median (Q2), upper quartile (Q3), and largest observation). A boxplot may also indicate which observations, if any, might be considered outliers. Boxplots can be useful to display differences between populations without making any assumptions of the underlying statistical distribution. The spacings between the different parts of the box help indicate the degree of dispersion (spread) and skewness in the data, and identify outliers. Boxplots can be drawn either horizontally or vertically. Box and whisker plots are also very useful when two or more data sets are being compared, Figure 1.



**Figure 1:** Boxplots illustration

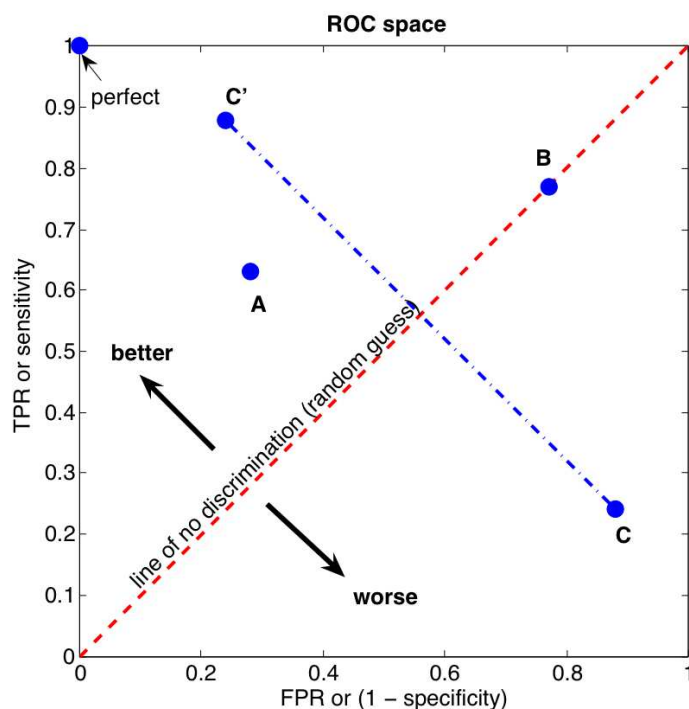
In a box plot:

- the ends of the box are the upper and lower quartiles, so the box spans the inter-quartile range (IQR)
- the median is marked by a line inside the box
- the whiskers are the two lines outside the box that extend to the highest and

lowest observations, if they are not outliers

- "Extreme" outliers, or those which lie more than three times the IQR to the left and right from the first and third quartiles respectively, are indicated by a star
- "Mild" outliers-that is, those observations which lies more than 1.5 times the IQR from the first and third quartile but are not also extreme outliers are indicated by the presence of a dot.

**Receiver Operating Characteristic (ROC):** A graphical representation of the relationship between the true positive rate (Sensitivity) and the false positive rate (100-Specificity) for different cut-off points. It is used to evaluate the efficacy of a tumor marker at different cut-off point. An ideal graph is the one giving the maximum area under the curve (AUC= 1).The best possible prediction method would yield a point in the upper left corner or coordinate (1.0) of the ROC space, representing 100% sensitivity (no false negatives) and 100% specificity (no false positives). The (1.0) point is also called a *perfect classification*. A completely random guess would give a point along a diagonal line (the so-called *line of no-discrimination*) from the left bottom to the top right corners. Therefore, the closer the ROC plot is to the upper left corner, the higher the overall accuracy of the test, Figure 2.

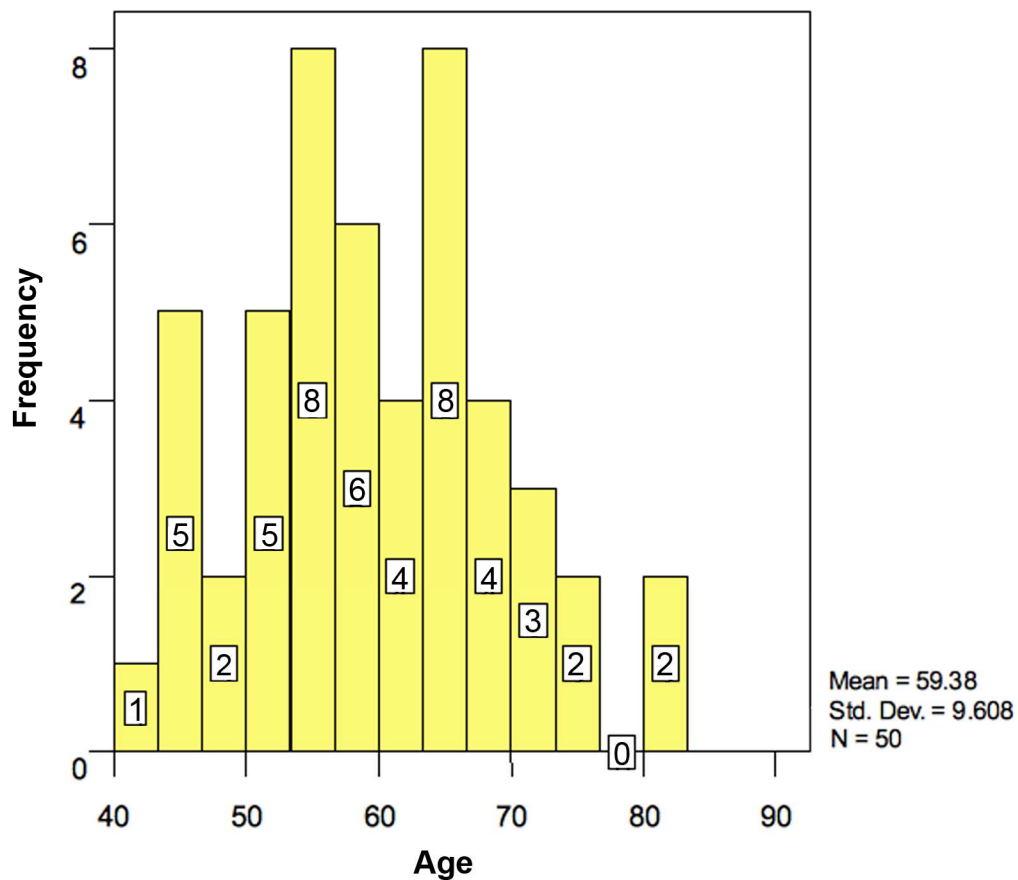


**Figure 2:** ROC curve illustration.

## 4. Results

### 4.1 Clinical evaluation

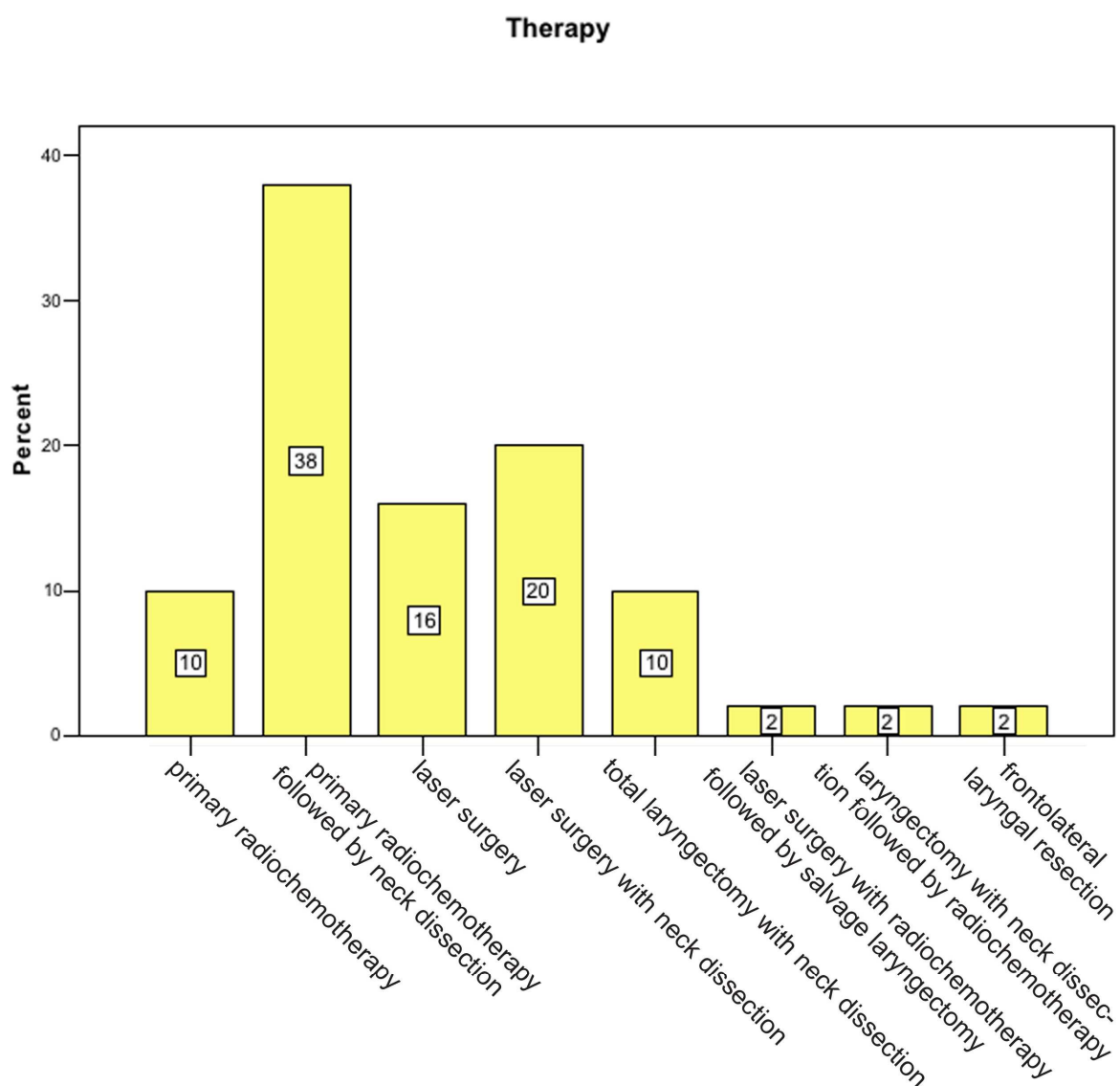
Fifty patients with head and neck squamous cell carcinoma (HNSCC), who were treated in the Dept. Otolaryngology, Head and Neck Surgery of Marburg, between 2003-2007, were entered into this evaluation. The patients ranged in age from 40 to 82 years, age distribution is seen in Figure 3.



**Figure 3:** Age distribution of 50 patients with laryngeal and hypopharyngeal SCC.

42 patients were men and 8, women. The anatomical sites of HNSCC were, the larynx (n=26), hypopharynx (n=24). Five patients had stage I disease, 8, stage II; 8, stage III; and 29, stage IV disease, according to the International Union against Cancer (UICC) staging system.

The treatment plan for the patients varies according to their clinical stage, for the early stage disease, Surgery in the form endoscopic laser surgery is the main stay of treatment for the majority of those patients. Advanced stage diseases were treated in a combined modality form (radio-chemotherapy and surgery) or radio-chemotherapy alone. Figure 4 illustrates the modality of treatment for patients.



**Figure 4:** The treatment plan for 50 patients with laryngeal and hypopharyngeal SCC according to their clinical stage.

During follow-up examination, only three patients (3/50) diagnosed to have a second primary tumor, oropharyngeal scc, hypopharyngeal scc, and bronchogenic carcinoma. All those patients diagnosed histologically.

According to the clinical course of the patients, we divide them into two major groups:

First group (n=32), all patients in this group had complete remission during one year follow-up, according to the line of management planned for each of them.

Second group (n=18), those patients with local residual disease, recurrence and/ or distant metastases. The organ distribution of metastases was illustrated in Table 1.

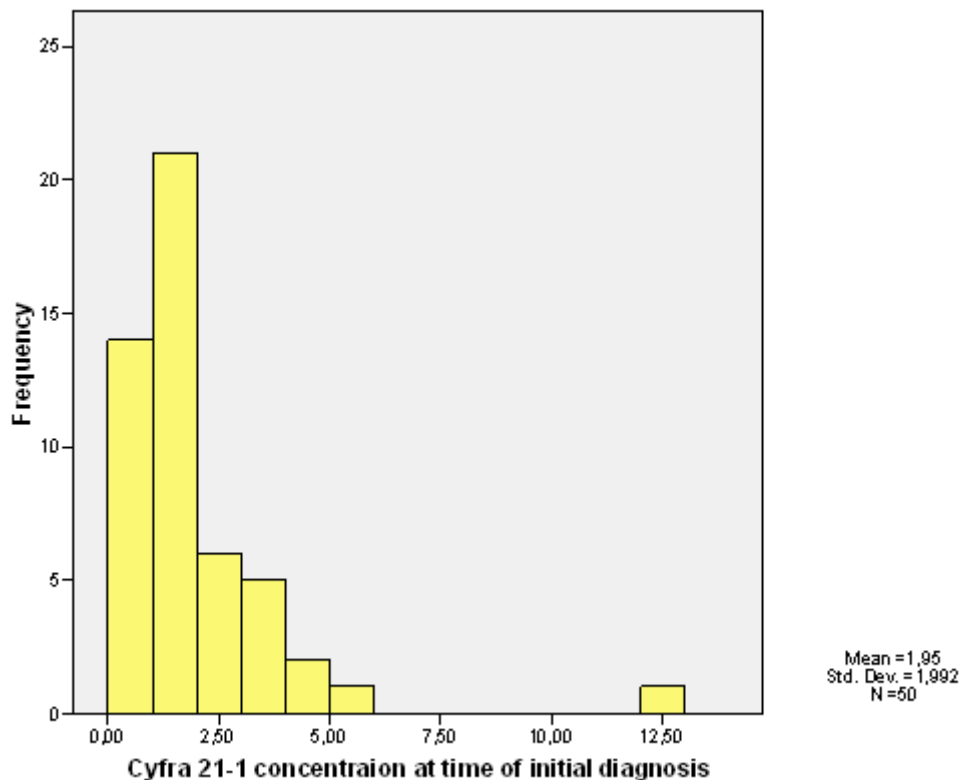
		Frequency
Valid	Complete remission	32/50
	Local residual disease	7/50
	Lung metastases	4/50
	Local residual disease and Lung metastases	1/50
	Local residual disease and bone metastases	1/50
	local residual disease and lung and brain metastases	1/50
	Local residual disease and Nasopharynx metastases	1/50
	lung and liver metastases	1/50
	Bone and skin metastases	1/50
	Suprarenal gland metastases	1/50
	Total	50/50

**Table 1:** The clinical course of 50 patients with laryngeal and hypopharyngeal SCC during the period of one year follow-up.



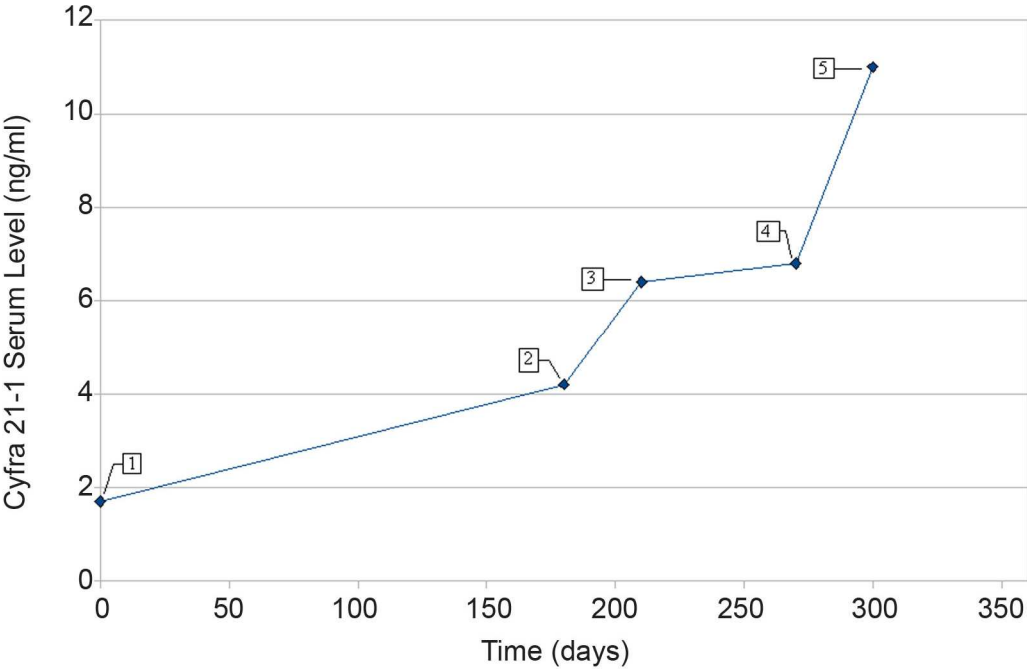
The calculated concentration of Cyfra 21-1 was expressed in ng/ml. In our hospital the cut-off level of 3.3 ng/ml was used but as I mentioned earlier the aim of the study not to depend on any reference value and our aim to look for abrupt rise in serum level of Cyfra 21-1 on follow-up.

A wide range of serum Cyfra 21-1 levels at the time of initial diagnosis were obtained from 0.32-13ng/ml, a mean=1.95ng/ml, a median=1.4ng/ml, Figure 5.

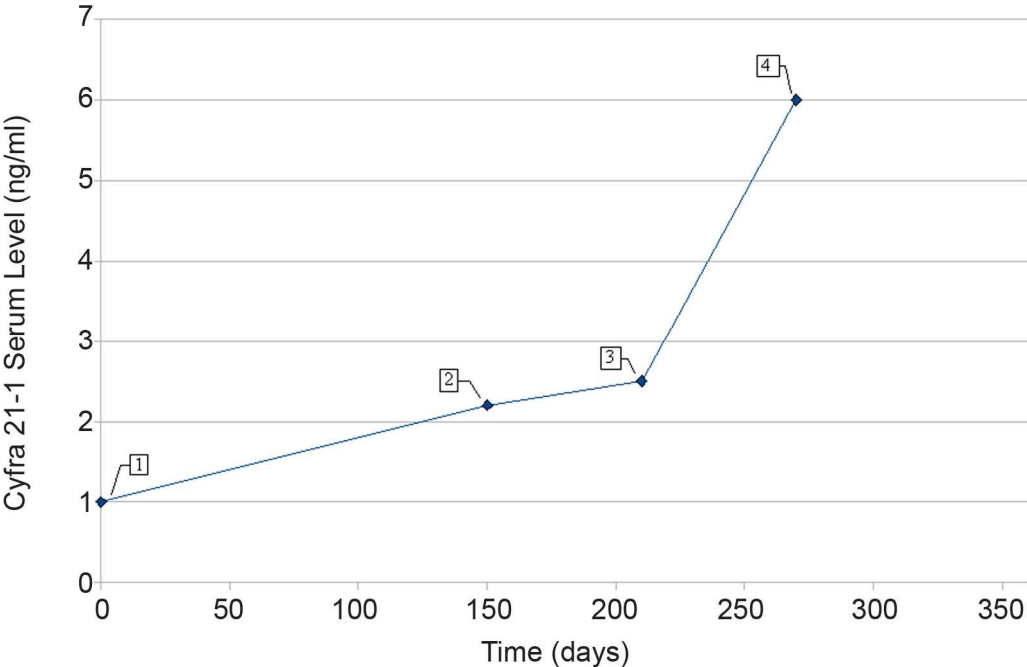


**Figure 5:** Cyfra 21-1 concentration at time of initial diagnosis in 50 patients with laryngeal and hypopharyngeal SCC.

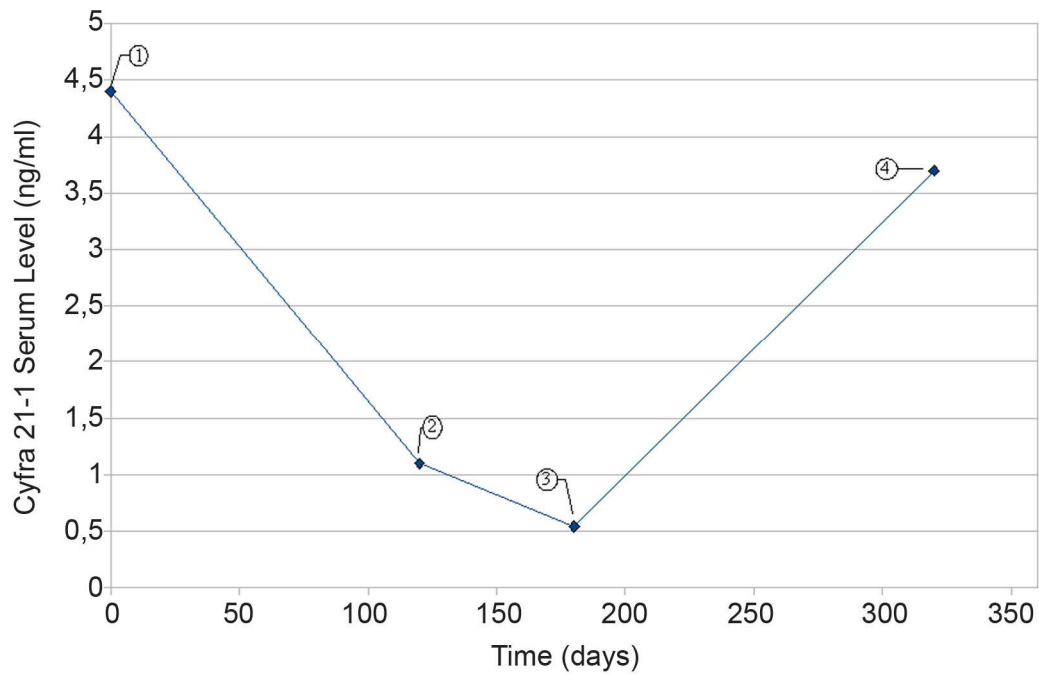
Figures 6-11 demonstrate graphically the serum level of Cyfra 21-1 in a selected group of our patients during the follow-up.



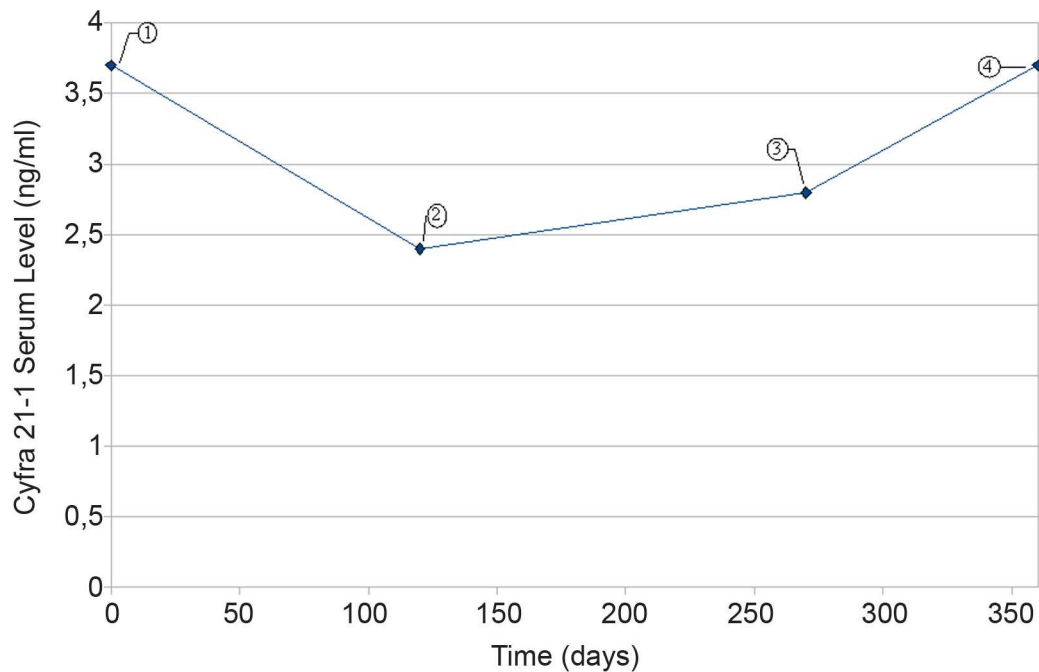
**Figure 6:** Serial measurements of the Cyfra 21-1 Serum Levels during clinical course of a patient with a T3N0M0 Laryngeal (Supraglottic) squamous cell carcinoma. Abrupt rise in the serum Cyfra 21-1 Level is seen on follow up, which is correlated with the clinical diagnosis of distant metastases. 1: primary diagnosis, 2: post-therapy, 3: before the clinical appearance of distant metastasis, 4: diagnosis of bone metastasis, 5: diagnosis of skin metastasis.



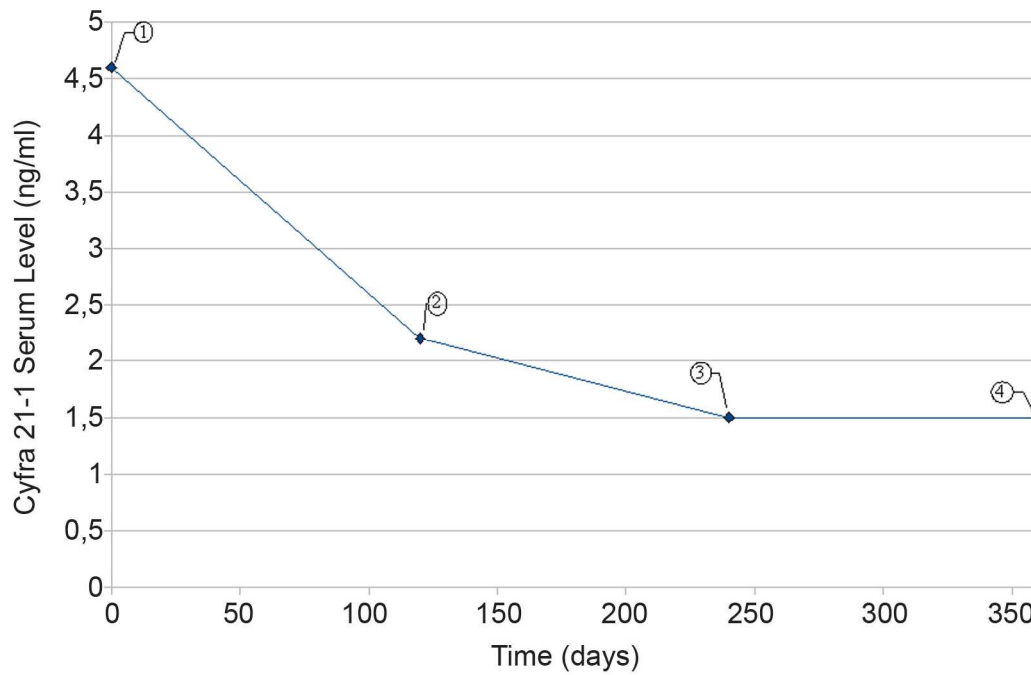
**Figure 7:** Serial measurements of the Cyfra 21-1 Serum Levels during clinical course of a patient with a T4N3CM0 Oro-Hypopharyngeal squamous cell Carcinoma. Abrupt rise in the Serum Level of Cyfra 21-1 is seen on follow up which is correlated with the clinical diagnosis of distant metastasis. 1: primary diagnosis, 2: post-therapy, 3: before clinical diagnosis of distant metastasis, 4: clinical diagnosis of distant metastasis (suprarenal gland).



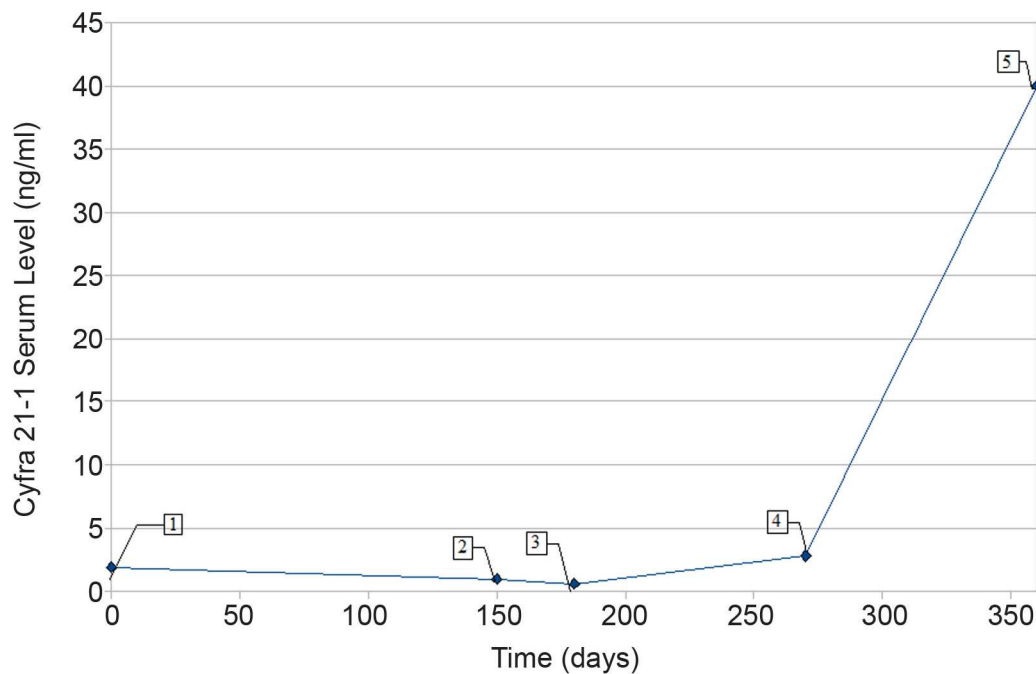
**Figure 8:** Serial measurements of the Cyfra 21-1 Serum Levels during clinical course of a patient with a T4N3M0 hypopharyngeal squamous cell carcinoma. Abrupt rise in the serum Level of Cyfra 21-1 on follow-up correlated with the diagnosis of local residual disease and lung metastases. 1: primary diagnosis, 2: post-therapy, 3: follow-up, 4: diagnosis of local residual disease and distant metastasis (lung).



**Figure 9:** Serial measurements of the Cyfra 21-1 Serum Levels during clinical course of a patient with a T2N2CM0 Laryngeal squamous cell carcinoma. Abrupt rise in the serum Level of Cyfra 21-1 on follow-up correlated with the diagnosis of local residual disease. 1: primary diagnosis, 2: post-therapy, 3: follow-up, 4: diagnosis of local residual disease.



**Figure 10:** Serial measurements of the Cyfra 21-1 Serum Levels during clinical course of a patient with a T3N2AM0 Hypopharyngeal squamous cell carcinoma. Absence of abrupt rise in the serum Level of Cyfra 21-1 on follow-up correlated with the clinical course of the disease (complete remission). 1: primary diagnosis, 2: post-therapy, 3: follow-up, 4: follow-up (complete remission).



**Figure 11:** Serial measurements of Cyfra 21-1 in a patient with T3N1M0 hypopharyngeal squamous cell carcinoma, shows abrupt rise in serum concentration (40ng/ml) during one-year follow-up, which correlated with clinical diagnosis of distant lung metastasis. 1: primary diagnosis, 2: post-therapy, 3&4: follow-up, 5: distant lung metastasis.

## 4.2 Analytical Evaluation

The correlation between the size of the primary tumor (T-status), and the serum concentration of Cyfra 21-1 at the time of initial diagnosis

Using the Jonckheere-Terpstra Test, no significant correlation exist between the primary tumor and the serum concentration of Cyfra 21-1 at the time of initial diagnosis (p value= 0.916), Table 2.

### Jonckheere-Terpstra Test.

	Cyfra_DX
Number of Levels in primary tumor	4
N	50
Observed J-T Statistic	434,500
Mean J-T Statistic	440,500
Std. Deviation of J-T Statistic	56,628
Std. J-T Statistic	-,106
Asymp. Sig. (2-tailed)	,916

**Table 2:** The correlation between serum levels of Cyfra 21-1at time of initial diagnosis and primary tumor using Jonckheere-Terpstra Test (p value=0.916).

There is a difference in the means of T-stages, but the Kruskal-Wallis H test shows, the difference in distribution is not significant (p value=0.698).

### Kruskal-Wallis Test

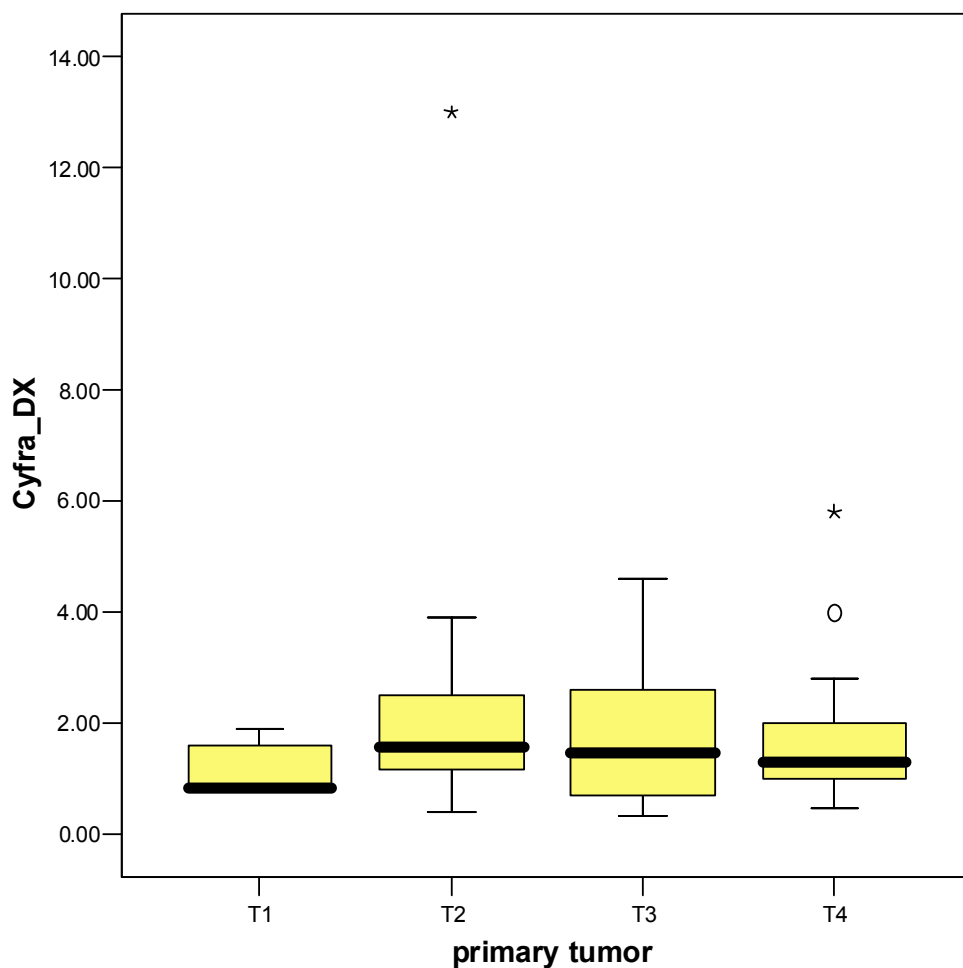
primary tumor	N	Mean Rank
Cyfra_ T1	5	20,40
DX T2	20	28,13
T3	12	23,88
T4	13	24,92
Total	50	

**Table 3:** The mean ranks for Cyfra 21-1 at different T-stages using Kruskal-Wallis test.

	Cyfra_DX
Chi-Square	1,432
df	3
Asymp. Sig.	,698

**Table 4:** Kruskal-Wallis test statistics for the grouping variable: Primary tumor (p value=0.698).

The median value for Cyfra 21-1 at time of initial diagnosis, T1=1.2, T2=1.6, T3=1.5, T4=1.3ng/ml, and the correlation between Cyfra 21/1 serum levels at time of initial diagnosis and primary tumor (T-status) using Boxplots were shown in Figure12.



**Figure 12:** The correlation between Cyfra 21-1 values at time of initial diagnosis and primary tumor size using Boxplots.

The correlation between the extent of Lymphogenic metastasis (N-status) and the serum concentration of Cyfra 21-1 at the time of initial diagnosis

Using the Jonckheere-Terpstra Test, no significant correlation exist between the extent of lymphogenic metastasis and the serum concentration of Cyfra 21-1 at the time of initial diagnosis (p value=0.424), Table 5.

#### Jonckheere-Terpstra Test.

	Cyfra_DX
Number of Levels in regional lymph node	6
N	50
Observed J-T Statistic	526,000
Mean J-T Statistic	480,000
Std. Deviation of J-T Statistic	57,539
Std. J-T Statistic	,799
Asymp. Sig. (2-tailed)	,424

**Table 5:** The correlation between the extent of Lymphogenic metastasis (N-status) and the serum concentration of Cyfra 21-1 at the time of initial diagnosis using Jonckheere-Terpstra Test (p value=0.424).

There are differences in the means between regional lymph node status, but the Kruskal Wallis Test shows, the difference in the distribution is not significant (p value=0.442).

#### Kruskal-Wallis Tes

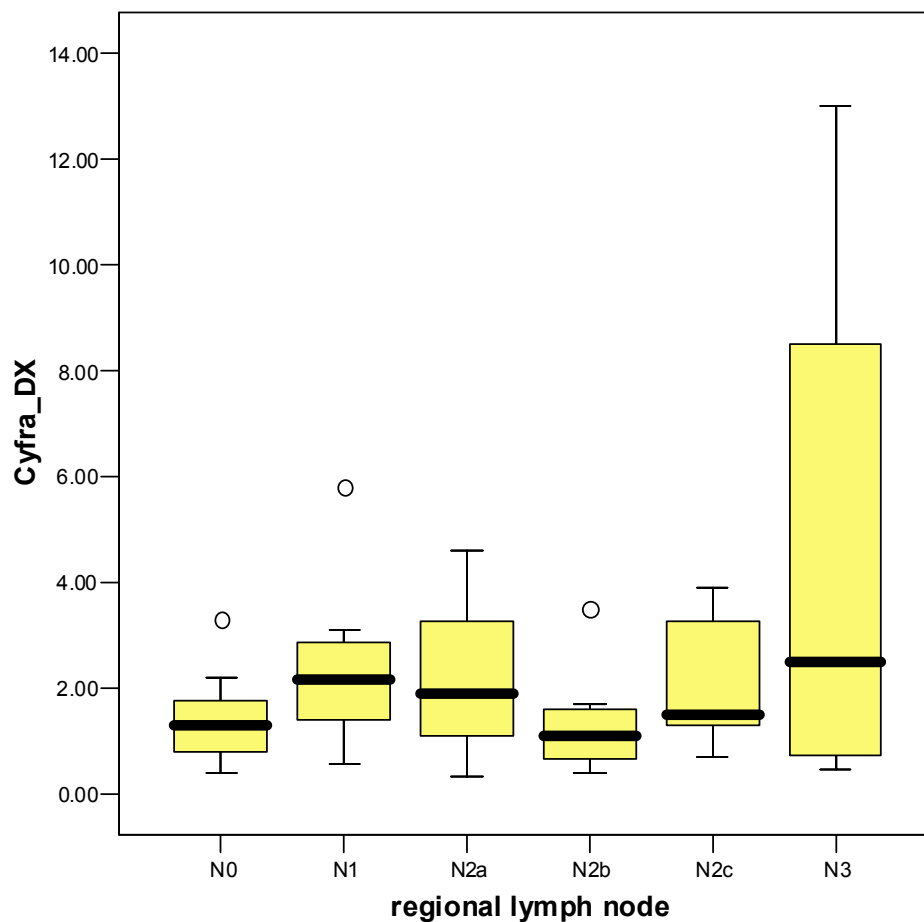
	regional lymph node	N	Mean Rank
Cyfra_DX	N0	19	23,11
	N1	8	31,50
	N2a	3	27,83
	N2b	9	19,00
	N2c	7	30,50
	N3	4	29,00
	Total	50	

**Table 6:** The mean ranks for Cyfra 21-1 at different N-status using Kruskal-Wallis test.

	Cyfra_DX
Chi-Square	4,794
df	5
Asymp. Sig.	,442

**Table 7:** Kruskal-Wallis test statistics for the grouping variable: N-status (p value=0.442).

The correlation between Cyfra 21-1 levels at time of initial diagnosis and lymphogenic status (N-status) using Boxplots were shown in Figure 13.



**Figure 13:** The correlation between Cyfra 21-1 values at time of initial diagnosis and regional lymph nodes (N-status) using Boxplots.



The correlation between the histological tumor grading and the serum concentration of Cyfra 21-1 at the time of initial diagnosis

Using the Jonckheere-Terpstra Test, no significant correlation exist between the histological tumor grading and the serum concentration of Cyfra 21-1 at the time of initial diagnosis (p value=0.462), Table 8.

#### Jonckheere-Terpstra Test.

	Cyfra_DX
Number of Levels in Grade	3
N	50
Observed J-T Statistic	242,500
Mean J-T Statistic	212,000
Std. Deviation of J-T Statistic	41,472
Std. J-T Statistic	,735
Asymp. Sig. (2-tailed)	,462

**Table 8:** The correlation between the histological tumor grading and the serum concentration of Cyfra 21-1 at the time of initial diagnosis (p value=0.462).

There is a difference in the mean ranks at different G-grades. But the Kruskal-Wallis Test shows that the difference in the distribution is not significant (p value=0.551).

#### Kruskal-Wallis Test

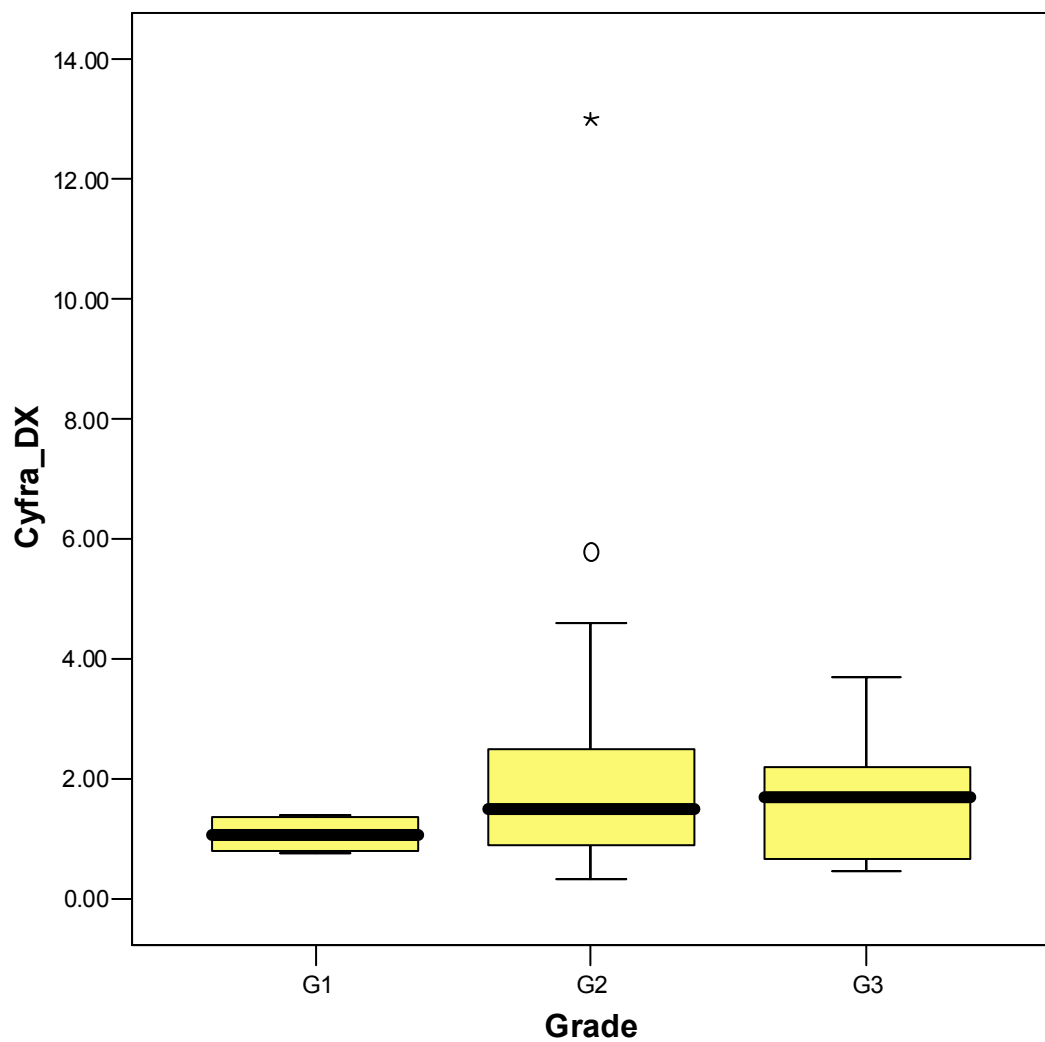
	Grade	N	Mean Rank
Cyfra_DX	G1	4	17,88
	G2	40	26,16
	G3	6	26,17
	Total	50	

**Table 9:** The mean ranks of Cyfra 21-1 at different G-grades using Kruskal-Wallis test.

	Cyfra_DX
Chi-Square	1,191
df	2
Asymp. Sig.	,551

**Table 10:** Kruskal-Wallis test statistics for the grouping variable: Grade (p value=0.551).

The correlation between Cyfra 21-1 levels at time of initial diagnosis and histological grade using Boxplots were also shown in Figure 14.



**Figure 14:** The correlation between Cyfra 21-1 values at time of initial diagnosis and histological grade using Boxplots.

The correlation between the clinical stage and the Cyfra 21-1 concentration at time of initial diagnosis

Using the Jonckheere-Terpstra Test, no significant correlation exist between the clinical stage and the Cyfra 21-1 concentration at the time of initial diagnosis (p value=0.504), Table 11.

#### Jonckheere-Terpstra Test

	Cyfra_DX
Number of Levels in Stage	4
N	50
Observed J-T Statistic	412,000
Mean J-T Statistic	376,500
Std. Deviation of J-T Statistic	53,123
Std. J-T Statistic	,668
Asymp. Sig. (2-tailed)	,504

**Table 11:** The correlation between the clinical stage and the Cyfra 21-1 concentration at the time of initial diagnosis (p value=0.504).

There is a difference in the mean ranks at different clinical stages. But, Kruskal-Wallis test statistics shows that the difference between the distribution is not significant (p value=0.845).

#### Kruskal-Wallis Test

	Stage	N	Mean Rank
Cyfra_DX	stage1	5	20,40
	stage2	8	24,63
	stage3	8	27,31
	stage4	29	26,12
	Total	50	

**Table 12:** The mean ranks for Cyfra 21-1 at different clinical stages using Kruskal-Wallis test.

The sensitivity and specificity of Cyfra 21-1 as follow-up marker for squamous cell carcinoma of the larynx and hypopharynx to detect local recurrence and distant metastases particularly lung metastases

Sensitivity: probability that a test result will be positive when the disease is present (true positive rate, expressed as percentage).

Specificity: probability that a test will be negative when the disease is not present (true negative rate, expressed as a percentage).

## 1. ROC Curve

### Complete sample

The table shows for all Cyfra 21-1 values in the sample sensitivity and 1-specificity, if we use thes Cyfra 21-1 values as cut-off points.

Complete remission defined as the state without disease and residual disease or metastases as disease state.

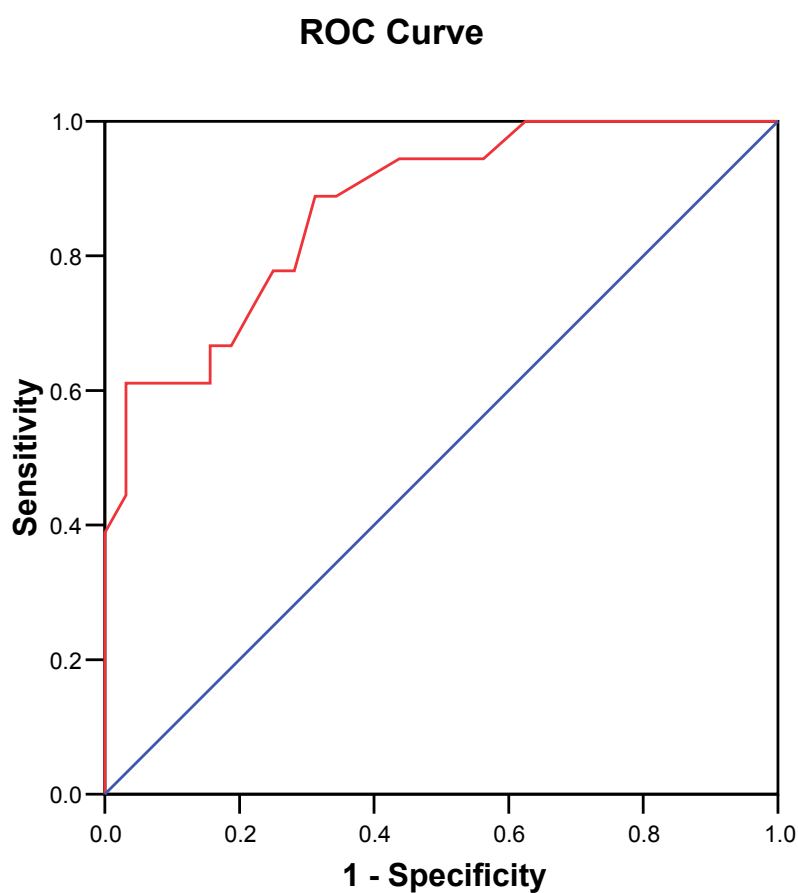
If all for Cyfra-values in the sample points with sensitivity as Y-coordinate and 1-specificity as X-coordinate are combined, a curve, called ROC-Curve, results

The ROC-curve should be located considerably above the diagonal line  $Y=X$

### Case Processing Summary

CLINICAL(b)	Valid N (listwise)
Positive(a)	18
Negative	32

**Table 13:** Case processing summary: The positive actual state is residual disease or metastases, negative state indicates complete remission.



Diagonal segments are produced by ties.

**Figure 15:** Receiver Operating Characteristic (ROC) curve for Cyfra 21-1 to detect local residual disease, recurrence and/ or distant metastases during follow-up of patients with laryngeal and hypopharyngeal SCC.

Area	Std. Error(a)	Asymptotic Sig.(b)	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.873	.050	.000	.775	.971

**Table 14:** Area under the Curve (AUC=0.873), according to the ROC curve, statistics may be biased. a: Under nonparametric assumption. b: Null hypothesis: True area=0.5.

## Coordinates of the Curve

Test Result Variable(s): Cyfra21-1 during follow-up.

Positive if Greater Than or Equal To(a)	Sensitivity	1 - Specificity
-.5100	1.000	1.000
.5650	1.000	.969
.6700	1.000	.938
.7500	1.000	.906
.8250	1.000	.875
.8550	1.000	.844
.9250	1.000	.813
.9950	1.000	.781
1.0500	1.000	.719
1.1500	1.000	.625
1.2500	.944	.563
1.3500	.944	.500
1.4500	.944	.438
1.5500	.889	.344
1.7000	.889	.313
1.8500	.778	.281
1.9500	.778	.250
2.0500	.722	.219
2.1500	.667	.188
2.2500	.667	.156
2.3500	.611	.156
2.4500	.611	.094
2.6000	.611	.063
3.0000	.611	.031
3.3500	.500	.031
3.5500	.444	.031
4.1000	.389	.000
5.2500	.333	.000
6.1000	.278	.000
7.4500	.222	.000
9.8500	.167	.000
21.0000	.111	.000
35.5000	.056	.000
41.0000	.000	.000

**Table 15:** ROC curve coordination: The smallest cut-off value is the minimum observed test value minus 1, and the largest cut-off value is the maximum observed test value plus 1. All the other cut-off values are the averages of two consecutive ordered observed test values.

The sensitivity and specificity of a test are dependent on the cut-off value that is used. When you select a higher cut-off level, the false positive fraction will decrease with increased specificity but on the other hand the true positive fraction and sensitivity will decrease, and when you select a lower cut-off value, then the true positive fraction and the sensitivity will increase. On the other hand, the true positive fraction will also increase, and therefore, the true negative fraction and specificity will decrease.

Cyfra 21-1 sensitivity and specificity at cut-off level of 3.3 were 61.1% and 96.9% respectively, to detect local recurrence and /or distant metastases as seen in Table 16.

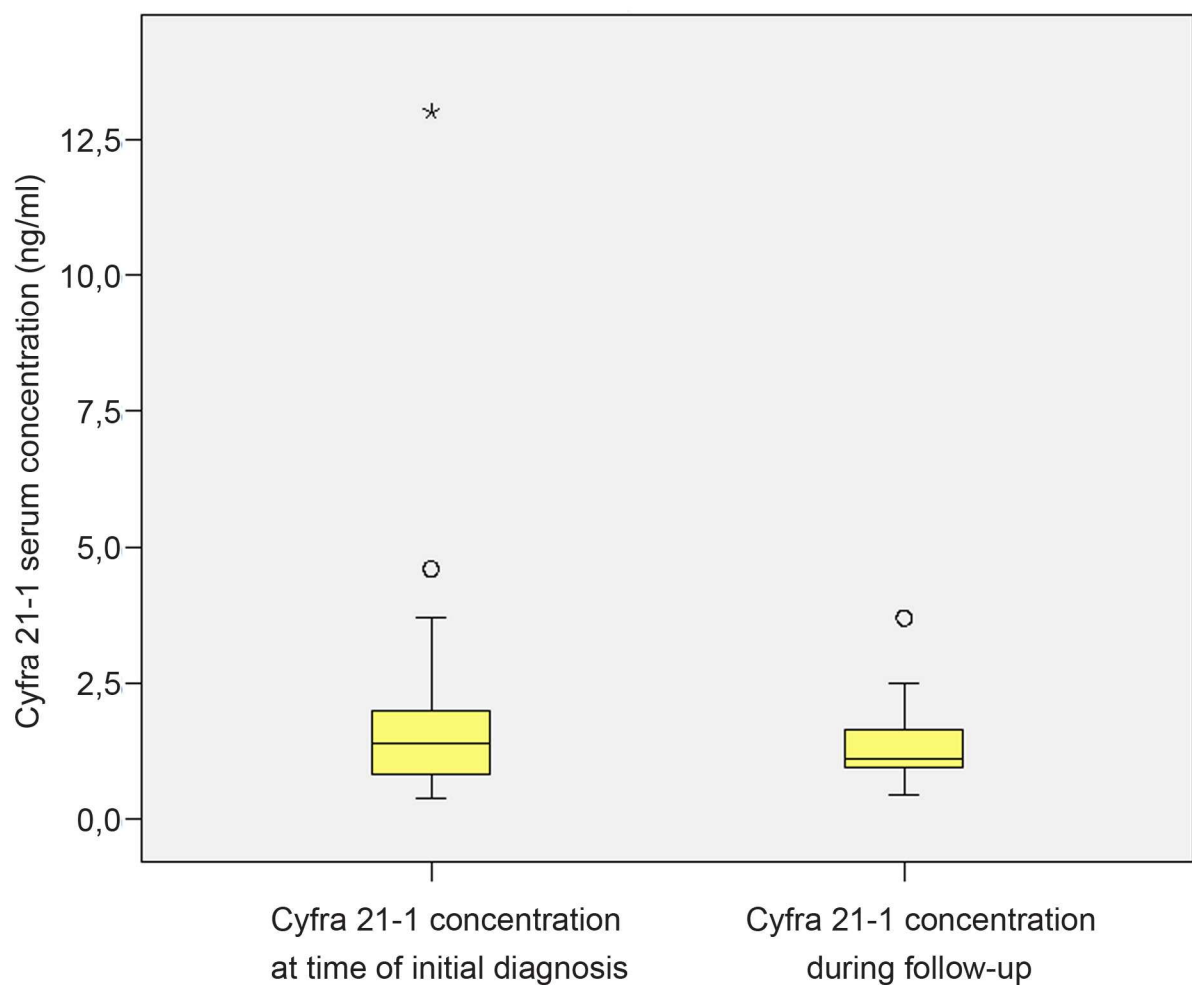
			CLINICAL		Total
			complete remission	residual disease or metastases	
Cyfra21-1 level	<3.3	Count	31	7	38
		% within CLINICAL	96.9%	38.9%	76.0%
	>=3.3	Count	1	11	12
		% within CLINICAL	3.1%	61.1%	24.0%
Total		Count	32	18	50
		% within CLINICAL	100.0%	100.0%	100.0%

**Table 16:** The sensitivity and specificity for Cyfra 21-1 at cut-off level of 3.3

## 2. Boxplots

For the patients with complete remission the Cyfra 21-1-concentration at diagnosis has a median of 1.4 and the median becomes smaller for last observed Cyfra 21-1 concentration (1.1) during follow-up.

The inter-quartile range (IQR), too, is smaller for the observed Cyfra 21-1 concentration on follow-up than for Cyfra 21-1-concentration at diagnosis. It is 1.24 at diagnosis and 0.71 during follow-up as illustrated using the Boxplots, Figure 16.



**Figure 16:** Comparison of Cyfra 21-1 concentration in serum between the time of initial diagnosis and follow-up in patients with complete remission.



For the patients with residual disease or metastases the Cyfra 21-1 concentration is higher for the observed value during follow-up, than at the time of diagnosis.

Median values for Cyfra 21-1 at the time of diagnosis: 1.4

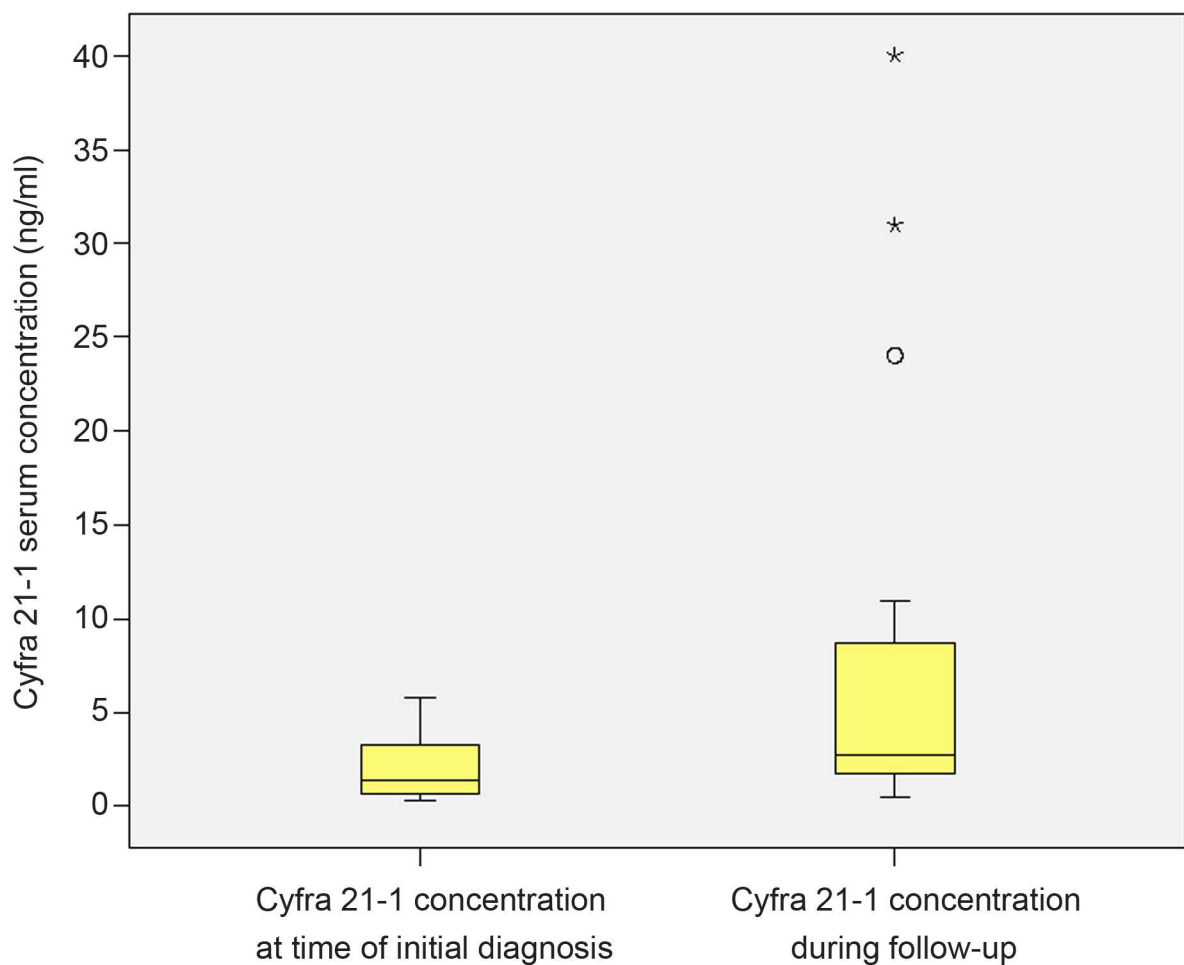
Median values for Cyfra 21-1 for the follow-up values: 2.7

The increase of the inter-quartile range is even stronger for follow-up values of Cyfra 21-1

Inter-quartile range at the time of diagnosis: 2.69

Inter-quartile range for the last observed value: 7.68

Boxplots show these changes, Figure 17.



**Figure 17:** Comparison of Cyfra 21-1 concentration in serum between the time of initial diagnosis and follow-up in patients with local recurrence, residual disease and/or distant metastases.

## **5. Discussion**

### **5.1. Follow-up of Patients with Head and Neck Squamous Cell Carcinoma**

The aims of cancer patients follow-up include the early detection of recurrences, metastases and secondary carcinomas; additionally, they involve adequate pain control therapy, as well as somatic-psychic and social rehabilitation and reintegration [15].

The interval between single tumor follow-up examinations depends largely on primary tumor location, the risk of developing a secondary carcinoma and the extent of the primary therapeutic intervention, as well as on the recurrence-free interval after initial treatment. About 90% of all local recurrences or regional metastases occur within the first two years after primary treatment [16]. Also, the risk of developing a secondary carcinoma increases every year after the initial treatment. A wide spread practice is to perform follow-up examinations during the first year after tumor therapy in intervals of 4 weeks, and to extend the intervals to 8 weeks during the second year, 3 months during the third year and 6 months during the fourth and fifth years [17].

In USA, according to study published by Marchant et al. [18], in the first year, they recommended a follow-up every month, in the second year, every two months, and in the third to fifth year, every 6 months. The guidelines of the German Society of Otorhinolaryngology, Head and Neck Surgery recommended two different follow-up schedules. For tumors having a low risk of recurrence or secondary occurrence in the upper aero-digestive tract, the recommended follow-up interval is 3 months during the first year, 4-6 months during the second year, every 6 months during the third-fifth years and after the fifth year, annually. For advanced-stage tumors, or after incomplete resection, control examinations are recommended at 6-week interval during the first year, at 3-month intervals during the second year, at 6-month intervals during the third-fifth years and, after the fifth year, annually [19].

The extensive and prolonged follow-up in those patients raise the question of benefits in term of survival, the survival time of patients with an initially advanced tumor stage can frequently not be prolonged despite intensive tumor follow-up.

Boysen et al. [20] concluded that, in spite of follow-up intervals of 2 or 3 months, long term survival could only be improved significantly within the first two years in patients suffering from laryngeal carcinomas that had been primarily irradiated.

According to study of Wolfensberger [21], a curative secondary treatment could only be performed in patients with a low T category without cervical lymph node metastases. The patients included in the evaluation were examined during the first two years 4 times per year, and during the third through fifth years, every six months.

Despite all the above mentioned results, other authors [18] favor intensive follow-up schedules in all patients with HNSCC.

Werner and Davis [22] recommended that in cases of circumscribed T1 and T2 upper aero-digestive tract tumors that were not primarily treated with neck dissection, to perform follow-up examinations during the first year every month, during the second year every two months, and during the third–fifth years, every three months. In these patients, regional recurrence can still be treated by neck dissection with curative intent. Also, such a follow-up is indicated in patients with laryngeal carcinoma that had been irradiated primarily. Tumor recurrences with regional lymph node metastases generally have a very poor prognosis even if they are diagnosed relatively early. These patients should be seen during the first two years every 3 months, during the third year, every 4 months and, afterwards, every 6 months, at the same time, any suspicion of the presence of recurrence or second primary carcinoma necessitate immediate examination and investigation accordingly.

The extent of follow-up examinations in patients with HNSCC is still a matter of discussions, as about 90% of patients with HNSCC will develop metastases or recurrence within the first two years after primary treatment [23]. And the expression of “tumor cure” is usually used after 5 years of tumor-free survival. Boysen et al. [20] recommend discontinuing follow-up examinations following the fifth year after primary treatment due to the fact that, according to their studies, therapy of a secondary primary carcinoma during this period does not lead to significant improvement in survival rate. In contrast, De Visscher and Manni [24] showed that the duration of follow-up should depend on the site and stage of the primary cancer. They demonstrated that a curative secondary therapy could be performed successfully in patients with a glottic laryngeal carcinoma in stages I and II up to 10 years after

primary treatment and, in stage III and IV, up to 2 years after primary treatment. With stage I and II supraglottic laryngeal carcinoma, curative resection could be performed successfully up to 3 years after primary treatment and, with stage III and IV, up to 7 years after primary treatment. Curative secondary therapy for subglottic cancer could be performed up to 2 years after primary treatment, carcinoma of the oral cavity and pharynx, up to 5 years after primary therapy.

The majority of authors [18, 21], recommended a lifelong follow-up in order to detect and treat secondary carcinomas with curative intention, especially for patients use tobacco.

Secondary carcinoma must be identified histologically [25], the chances of developing a secondary carcinoma between 10-20% in patients suffering from malignant tumors of the head and neck and the yearly incidence amount to 3-7%. There is a clear tendency for secondary carcinoma to manifest in the aero-digestive tract if the primary tumor was located in the oral cavity, oropharynx or the hypopharynx [26]. The decisive factor for the chances of surviving a second carcinoma is site. Secondary carcinomas located in the lung or esophagus nearly always have a very unfavorable prognosis. In contrast, secondary carcinoma in the region of the oral cavity or the larynx can be cured, if they are diagnosed and treated early [22].

Clinical studies show an incidence of distant metastases in patients with HNSCC varies between 4-26%. In contrast, autopsy examinations reveal a higher incidence, with a value more than 40% [23]. The frequency of distant metastases at first presentation is between 1.5% and 16.8%. The initial diagnosis occur typically 9-12 months after initial tumor identification, and in 84% of the cases, it occur within the first two years. Generally, all regions of the body can be affected by distant metastases of squamous cell carcinoma of the head and neck. Distant metastases of HNSCC are mainly influenced by the location of the primary tumor and the initial T and N stage [24]. The most frequent sites of distant metastases are the lung, liver, and bones respectively [25]. The average survival in patients with distant metastases is between 4.3-7.3 months [26], as a result, these patients are generally considered terminally ill and palliative treatment only provided.

Bier et al. [17] recommended a yearly routinely performed pan-endoscopy, as the most reliable diagnostic procedure for detection of secondary carcinomas, as well as recurrence or metastases of the upper aero-digestive tract. In contrast, many other authors not considering pan-endoscopy performed yearly as part of standard follow-up. Pan-endoscopy, generally done under general anesthesia, which creates additional risk for patients due to co-morbidities. Recently, the use of ultra-thin transnasal esophagoscopy under local anesthesia, obviate the need for general anesthesia [22].

B-mode sonography is considered the investigation of choice for the diagnosis of lymph node metastases of the head and neck with a sensitivity of more than 70% and a specificity of nearly 100%, and it can be accompanied by U/S-assisted aspiration cytology [27]. Some authors recommend a sonographic examination of the neck at each follow-up visit during the first two years [17].

The purpose of the yearly chest X-ray performed routinely by many authors is to diagnose secondary carcinoma or metastases from a head or neck primary cancer to the lungs [17, 24]. However, conventional chest X-ray, do not detect carcinoma of the lung at an early stage. A prospective study evaluated by Rainer et al. [28] showed that only 29% of pulmonary metastases or secondary carcinoma diagnosed in a thoracic CT-scan could also be detected with ordinary chest AP and lateral x-rays.

For oncological follow-up during the first 5 years, Bier et al. [17] recommended routine performance of yearly sonography of the abdomen. Given the course of the disease, abdominal distant metastasis occurs very rarely and, when they do occur, offer few therapeutic options. Because of this, the usefulness of routinely performed abdominal sonography in asymptomatic patients must be questioned. Some authors [22] do not recommend sonography of the epigastric region, nor do they recommend abdominal CT or MRI scans.

Using scintigraphy of the skeleton (bone scan), it is possible to identify bony neoplasms smaller than 1 cm earlier than with conventional X-rays [29]. Due to the high number of false-positive results, further diagnostic clarification is necessary using ordinary X-ray, CT, or MRI scans [30].

Many patients today are treated in a multidisciplinary manner, where the patient follow-up is shared between the head and neck surgeon and the radiation oncologist. Immediately after surgery, of course, the surgeon must perform the follow-up, once the patient is through the period of potential postoperative complications or other postoperative management requirements, however, other specialist can check for tumor recurrence. Typically, in the first year after surgery, at approximately the third follow-up month, subsequent visits can be rotated on a one-month basis between the radiation therapist and the head and neck surgeon. When patients with advanced cancer receive radio-chemotherapy as part of their treatment, medical oncologist will also be involved. They are expert in the evaluation and treatment of distant metastases [22].

## **5.2 Serum tumor markers in head and neck cancer**

Serum tumor markers are defined as proteins with carbohydrate or lipid domains that are found circulating in blood and/or various other body fluids. Their appearance and changing concentrations are associated with the development and growth of malignant tumors [31]. These serum tumor markers have been used as prognostic markers for tumor recurrence or metastasis [32], e.g., CEA, SCCAg, Cyfra 21-1, TPS, etc. The levels of serum tumor markers reflect tumor burden and are not sensitive enough to be used for screening and early diagnosis of primary cancer. By contrast, the role of serum tumor markers is established in the diagnosis of recurrent disease and in the evaluation of response to treatment [33].

Recently, the role of tumor markers in management of head and neck cancer has received increasing attention. Serum or biochemical tumor markers constitute a variety of heterogenous substances that show quantitative changes during tumor development. The origin of these substances could be from the tumor itself, as is the case with ectopic hormone secretion, oncofetal antigens, or excess of some metabolic products of the neoplastic cells. Alternatively, some markers may be produced by the host in response to the developing tumor [34]. A perfect tumor marker should ideally have certain characteristics. First, the marker used should have a high degree of sensitivity. This is defined as the frequency of elevation (or depression) of a certain marker in a group of patients with malignancy (positive

results in a positive population). Second, the marker used should have a high degree of specificity. This is defined as percentage of negative results in normal population. Third, the level of a marker should correlate with tumor burden [34]. Consequently, correlation with the stage of the disease, both locally and regionally would be expected. Additionally, if a pretreatment value of a marker is obtained as a baseline for a patient, then serial samples obtained in the post-therapy period could be used to monitor the patient response to treatment. If therapy has been successful with a reduction of tumor mass, the serum level should decrease, and remain low in the absence of recurrence and/or distant metastases. If, however, there is a persistent rise in the level of the marker, this would suggest recurrence, residual disease, occult metastatic lesion, or the development of a second primary [34]. Ideally, this elevation should be early enough in the course of the development of such events to permit earlier detection and prompt management. Last, the level of a marker should correlate with tumor biological behavior and provide an additional prognostic index. For instance, marked elevations of pretreatment levels of a marker or failure to decline to normal levels after therapy may be indicators of poor prognosis [35].

Potential uses of serum markers include monitoring reduction of tumor mass after therapy, detecting recurrence or metastases during follow-up, screening populations at risk of developing a certain cancer, predicting patient prognosis based on initial values of a marker, or changes in serum levels after therapy [34].

### Squamous Cell Carcinom Antigen (SCC-Ag)

In 1977, Kato and Torigoe [36] described a tumor associated antigen (TA-4), in the serum of patients with squamous carcinoma of uterine cervix. Using radio-immunoassay, circulating antigen activity was detected in 27 of 35 (77%) patients with cervical squamous cell carcinoma. The eight patients with squamous cell carcinoma of the cervix who failed to demonstrate detectable antigen activity had early clinical stages, while all cases with advanced stage of the disease showed detectable levels. Changes in serum antigen levels reflected progression or regression of the disease [37]. The proportion of positive cases was higher in patients with metastasis than in those without metastasis. Elevated pretreatment

levels of serum TA-4 correlated with the extent of the disease, and patients with marked elevation (more than 15mU/ml) showed significantly worse prognosis [38].

This antigen is clearly released from the squamous cell carcinoma tissue [38], however, it is not keratin, studies of TA-4 indicated that it is not a single substance, but a series of proteins with a common antigenic determinant and a molecular weight of approximately 48,000 daltons [39]. A standard radioimmunoassay kit (SCC-RIA), developed by further purification of TA-4, became available for research and was reported to have a high degree of specificity. This accelerated investigations of the clinical usefulness of the SCC-antigen assay in patients with squamous cell carcinoma of other primary sites, including lung and esophagus. Johnson et al. [40] demonstrated elevated pre-treatment SCC-Ag levels in 27 of 60 (45%) patients with head and neck squamous cell carcinoma. Eibling and colleagues [41] conclude that the routine evaluation of pretreatment levels of SCC-Ag failed to show elevated levels in more than 50% of patients with squamous cell carcinoma of the head and neck regardless of patient age, sex, clinical stage, or tumor differentiation, however, within the group of patients with elevated levels, pretreatment levels correlated with tumor burden, and post-therapy levels correlated with the clinical course of the disease. A recent prospective study of a panel of tumor markers as prognostic factors in patients with squamous cell carcinoma of head and neck, showed that SCC-Ag has the lowest sensitivity (14%) in comparison with other markers used, with no particular correlation with age, gender, grade or cancer stage [42].

### Carcinoembryonic Antigen (CEA)

Carcinoembryonic Antigen (CEA) was first described by Gold and Freedman [43] as a new tumor specific antigen for colorectal cancer. Silverman et al. [44] determined CEA levels for 439 patients with squamous cell carcinoma of the head and neck. Both the incidence and magnitude of CEA elevations correlated with the clinical stage of the disease, in addition elevated levels declined to within the normal range after tumor resection. However, when patients with advanced stage excluded, similar levels of the antigen were found in tumor bearing patients, tumor-free-patients, and smokers, limiting the usefulness of this tumor marker in early detection of carcinoma of the head and neck. The higher levels of CEA in chronic smokers indicated that the



definition of abnormal CEA levels depend on the control population used. Schneider et al. [45] found elevated levels of CEA in 40 of 85 patients with head and neck cancer, but no correlation could be demonstrated between the level of CEA and site or stage of the disease. Likewise, AL-Sarraf and associates [46] found no correlation between levels of CEA and the site or morphology of cancers of the head and neck but levels appear to correlate with response to therapy. It was concluded that although CEA levels were not predictive of survival, and not likely to assist in prognosis after therapy, they did correlate with tumor burden and may have adjuvant value in monitoring tumor response to therapy. Recent studies showed a correlation between M status (distant metastases) and CEA, finding CEA a reliable tool in detecting distant metastases with no particular application in squamous cell carcinoma [42].

## Ferritin

Ferritins are generally regarded as isometric proteins that play a key role in iron storage and metabolism. The majority of ferritin is found in tissues, very small amounts are found in serum of healthy individuals [47]. Several causes lead to increase serum levels of ferritin in cancer patients, their relative contribution may vary in different cancers and in different stages of any given disease. These include (1) abnormalities in hemopoiesis and iron metabolism leading to abnormal levels of glycosylated ferritin similar to normal serum ferritin ; (2) nonspecific tissue damage, leading to elevation of an iron-containing ,glycosylated ferritin ; (3)direct secretion by the tumor, this latter source perhaps offer the most interest in ferritin as a tumor marker [48] .

Maxim and Veltri [48] reported elevated levels of serum ferritin in head and neck cancer patients and smokers. Serum ferritin levels were significantly lower in stage I and II disease than in patients with stage III and IV. The ferritin levels became elevated not only as the tumor increased in size from T1 to T2, but also when there was clinical evidence of spread to regional lymph nodes, N1 through N3. In patients with no evidence of clinical disease 5 years after treatment, the ferritin level had essentially returned to normal. After completion of successful therapy, a significant decline in serum ferritin levels occurred 5 months later. Furthermore, ferritin levels

showed a tendency to increase or remain high in patients with poor prognosis and to decrease in those with favorable prognosis.

#### Glycoprotein Cancer Associated Antigens: CA-50 and CA-19-9

CA-50 and CA-19-9 were identified by monoclonal antibodies defining different tumor-associated carbohydrate antigens on cell membranes of various malignant neoplasms [49]. Serum levels of CA-50 and CA-19-9 were found elevated in patients with head and neck neoplasms. Elevated CA-50 was found in 27% of patients with squamous cell carcinoma, 33% of patients with malignant salivary neoplasms, whereas only 2 of 21 benign salivary neoplasms had elevated serum values. Levels of CA-19-9, displayed a similar distribution to CA-50 but was less sensitive. For squamous cell carcinoma, no correlation between tumor stage or grade and serum levels was detected for any of markers. For malignant salivary gland tumors, however, CA-50 was particularly sensitive, and patients with mucoepidermoid carcinomas showed the highest incidence of elevated values. It was concluded that neither of these markers deserved a place in the routine examinations of patients with head and neck cancer [49].

#### Serum Enzymes

The serum levels of various enzymes have been studied in different cancer patients and have been shown to be of as significance in evaluation of such patients [50]. Several mechanisms may be responsible for quantitative changes in the serum level of various enzymes in cancer patients. The rapid cell turnover characteristic of malignancy may be associated with liberation of intracellular enzymes into the extracellular fluids and the circulation [50]. Several studies showed increased serum phosphohexose isomerase (PHI) in head and neck cancer. Goel et al. [51] reported more than two fold increase in the mean serum activity of PHI in 28 patients with head and neck cancer. Increase in serum PHI activity was directly proportional to the clinical stage of disease. A progressive decline in serum PHI levels was observed with successful therapy. Harbans et al. [52] evaluated the level of serum adenosine deaminase (ADA) in 40 patients with head and neck cancer. The mean value of ADA

in such patients was significantly higher compared with controls. The serum levels correlated with tumor burden, as well as response to therapy.

## Immunoglobulins

Immunoglobulin A (IgA), Brown et al. [53] found a significant increase in of IgA in both serum and saliva of 102 patients with oral carcinoma. While the serum level remained elevated through the course of the disease, salivary IgA returned to normal with cure and re-elevated with recurrence. Katz et al. [54] showed elevated levels of serum IgA in patients with head and neck cancer. This increase was specific to IgA, and elevated levels of IgG and IgM were not seen in as great a frequency; and, therefore, IgA/IgM and IgA/IgG ratios were noted to be increased in this patient population. Other studies reported a direct relation between serum IgA levels and tumor stage and a decrease in geometric mean titers after successful treatment. This was not confirmed in a recent study by Veltri et al. [55] who reported a consistent pre and post-therapy elevation of serum IgA in head and neck cancer patients. The significance of the elevated serum IgA and its possible relevance to immune regulation in head and neck cancer patients was reported. Excess IgA may represent a tumor specific antibody response, especially in those tumors involving mucosal tissues and secretory epithelium [54, 55]. These antibodies, however, could act as “blocking agents” (i.e., excess IgA may combine with tumor-associated antigens) thereby rendering them ineffective as antigenic stimuli [54]. Indeed, preliminary data suggested that the higher the serum IgA level, the poorer the prognosis in patients with carcinoma of the head and neck [56]. A serum immunoglobulin prognostic index (SIPI) using multiple immunoglobulin was suggested. A positive SIPI indicated a relative excess of IgA over IgE and IgD and was associated with increased failure rates. The overall accuracy of this formula in predicting the outcome of treatment was 60%, with higher predictive value when SIPI score was either highly positive or negative. The accuracy of SIPI score was further enhanced when combined with patient clinical parameters such as stage, age, and lymphocyte count [57].

## Immune Complexes (ICS)

Increased circulating immune complexes were reported in the sera of patients with a variety of tumor [58]. The incidence and levels of immune complexes (ICS) were significantly higher in patients with advanced local disease, as well as patients with metastatic disease. It was demonstrated that almost 75% of patients with carcinoma of the head and neck had elevated soluble ICS in their sera and a tendency for these factors to remain elevated well into the post-therapy period [55]. Head and neck cancer patients often demonstrate a significant depression of cellular-mediated immunity (CMI) that persist even after therapy is completed. Such immune depressed status has been studied using variable parameters. Veltri et al. [55] assessed variable humoral and cellular immune responses in patients with head and neck cancer and defined the possible role of ICS in directly modulating CMI in such patients. The results implied that the prolonged immune depressed status of head and neck cancer patients may be related, in part, to the presence of ICS or IC-like substance in their serum. The persistently immune-depressed status in turn may be related to the incidence of recurrence, development of second primaries, and other immune-pathological sequel observed in patients with head and neck cancer.

## Serum Glycoproteins

The serum levels of a variety of normally occurring glycoproteins have been shown to correlate significantly with tumor extent, clinical course, and response to therapy in patients with various malignancies [59]. Wolf et al. [60] demonstrated that serum levels of some acute-phase reactant, namely, haptoglobin, alpha-1-acid glycoprotein, and alpha-1-antitrypsin showed differing relations with tumor extent and clinical stage in head and neck cancer. Levels of alpha-1-antitrypsin and alpha-1-acid glycoprotein increased progressively with increasing tumor extent and correlated significantly with tumor stage at the primary site and with nodal status. Serum haptoglobin levels were significantly elevated in all tumor stages but did not vary by stage. Serum levels of these glycoprotein were significantly lower in cured patients than in patients with either untreated or recurrent carcinoma. It was suggested that decline in the levels of these proteins could be expected with tumor ablation. On the

other hand, levels of alpha-2 HS-glycoprotein, pre-albumin, and albumin were significantly lower in cancer patients when compared to controls.

### Erythrocyte polyamines

Polyamines are low molecular, highly charged organic cations that are ubiquitous in nature [61]. The naturally occurring aliphatic polyamines, putrescine, spermidine, and spermine, are found in all types of cells [62]. It has been suggested that they are closely involved in cell growth and possibly in the regulation of RNA-dependent protein synthesis. Several investigators have confirmed that polyamines are elevated in the extracellular fluids in patients with cancer [61]. Shideler et al. [62] measured erythrocyte polyamines in 29 previously untreated patients with head and neck cancer. Elevated levels of erythrocyte spermidine and/or spermine were found in 31% of these patients when compared to reference ranges determined for normal subjects. An increase in both erythrocyte spermidine and spermine was observed with advanced tumor stage, the greatest difference being between stages I and IV for each of the polyamines. Moreover, the erythrocyte spermidine concentration decreased significantly with therapy. Shideler and co-workers concluded that although measurement of erythrocyte polyamines was not a sensitive screening indicator for patients with head and neck cancer, it correlated with tumor size and consistently decreased after tumor treatment.

### Prostaglandins and Prostacyclins

It has been suggested that prostaglandins of the E series have an important role in tumor growth and enhancement of carcinogenesis. Increased synthesis of prostaglandins has been reported in medullary thyroid tumors, bronchial carcinoma, hypernephroma, and carcinoma of the breast [63]. Prostacyclin is known primarily for its platelet anti-aggregative effect, and is produced under normal conditions by endothelial cells. The hemostatic balance mechanisms, particularly those due to interaction between prostacyclin (PGI<sub>2</sub>) formed by the vessel wall, and thromboxane generated from platelets, are of key importance in seeding of metastases [64]. Patients with maxillofacial cancer have significantly elevated plasma levels of (6-oxo-

PGI 1-alpha) a stable metabolite of PGI 2. Such elevated levels, decline after successful surgical intervention. Patients with local recurrence showed an increase in plasma (6-oxo-PGF 1-alpha) that was related to the extent of the disease [65]. It was noted, however, that increased (6-oxo-PGF 1-alpha) was found particularly in poorly vascularized squamous cell carcinomas. Such findings suggested that the elevated levels of PGI 2 and its metabolites may not be due to overproduction by tumors, but rather represent a host reaction to tumor growth designed to limit disease spread. Also, peri-tumoral inflammatory reaction, mediated by prostaglandins (PGE2 and PGI2), may provide further explanation for elevated levels in such patients [65]. Definite conclusions about the clinical relevance of prostaglandins, prostacyclins, and their metabolites in head and neck cancer not yet established.

### **5.2.1 Serum Cytokeratin Fragments**

Cytokeratins (Ck), belonging to the intermediate filament (IF) family of proteins, are particularly useful tools for diagnosis in oncology. At present, at least 37 different human CK have been identified of which CK 8, 18, and 19 are the most abundant in simple epithelial cells [8]. It has been observed that when malignant cells disintegrate, partially degraded CK fragments are released into the circulation and can be quantified using various commercially available specific serological assays [9]. CK fragments released from proliferating or apoptotic cells have proved to be useful marker for epithelial malignancies, and their subsequent release occurs during the intermediate events in apoptosis [10]. According to these investigators, the clinical value of determining soluble CK protein fragments in the body fluid lies in the early detection of recurrence and the fast assessment of the efficacy of response to therapy in epithelial cell carcinomas [9, 10].

The three most frequently used CK which are being evaluated as serum markers for their utility in clinical applications are tissue polypeptide antigen (TPA), tissue polypeptide specific antigen (TPS), and cytokeratin fragments 21-1 (Cyfra 21-1). Assays for TPA measure CK 8, 18, and 19. Assays for TPS and Cyfra 21-1 are more specific and measure CK 18 and CK 19 levels, respectively [10]. Cyfra 21-1 and TPS have been shown to be highly sensitive and specific for their prognostic value in head and neck malignancies [10, 14].

## Cytokeratins

Amongst the three cytoskeletal systems found in eukaryotic cells, the intermediate filament (IF) protein family is most complex. Depending on their polymerization properties and tissue specificity they are divided into six subtypes. Intermediate filaments of type I and type II are cytokeratins [66].

CK make up the largest subgroup of IF proteins and represent the most abundant proteins in epithelial cells. Their expression is site specific and differentiation dependent [66, 67]. The epithelial CK are closely related-both biochemically and immunologically. At present, more than 60 CK genes have been identified from the human genome sequence; of them 54 are functional genes [8]. Out of these, 37 different human CK genes have been identified. Ck are sub-grouped into type I (40-56.5 kDa) and type II (53-67 kDa) CK. Type I are acidic while Type II are basic CK [66]. Depending on their tissue expression pattern, they have been grouped into simple epithelia specific CK (CK7, 8, 18, 19, 20) and stratified epithelia specific CK (CK 4, 5, 13, 14, etc.) [68]. The most abundant epithelial CK are CK 8, 18, 19 [69]. CK like all other IF demonstrate high resistance to detergent action and to high and low ionic salt concentrations. Protein structures of CK consist of a central alpha helical rod domain, flanked on either side by amino terminal (head) domain and carboxy terminal (tail) domain. The alpha helical rod domain is a highly conserved region amongst all IF, while the head and the tail domains impart differential characteristics like molecular weights, isoelectric point, and antigenicity [70].

The primary function of CK is to protect epithelial cell from mechanical and non-mechanical stresses that resulting in cell death. Other emerging functions include roles in cell signaling, the stress response, apoptosis, and other tissue specific functions. The involvement of CK in a number of human diseases is now established and integrates well with the evidence gathered from transgenic mouse models [67]. Cytokeratins undergo several post-translational modifications which are important from the regulatory, mechanistic and functional perspectives. Most of the post translational modifications occur in the head and the tail domains. Phosphorylation, glycosylation, transglutamination and proteolytic degradation are some of the important post-translational modifications of these proteins and have been associated with some regulatory functions e.g., sub-cellular localization and

interaction with other cytoplasmic proteins [71]. These modifications influence the biological activity of the filaments resulting in increased solubility and filament reorganization [72].

CK expression pattern in the malignant cells is usually retained from the cell of origin, and therefore CK are being used in tumor typing, e.g., to discriminate epithelial cells from mesenchymal or lymphoid cells and also to establish the identity of secondary tumors in case of unknown primaries [72]. Reports from different laboratories have shown that, CK expression may be altered after malignant transformation, the change being consistent for that tumor type [72, 73]. Simple epithelia specific CK 8, 18 and 19 are normally not expressed in oral tissues, however, they are expressed in oral SCC [74]. Aberrant expression of CK 8 and 18 is the most common change in human oral cancer which has been reported by many groups [75]. Ck 8 and 18 expression has also been correlated with invasiveness of the tumor margin and poor prognosis of human oral SCC [76]. Cytokeratin deposition has been reported to occur in the necrotic regions intratumorally because of increased proteolytic activity in these cells [77]. The possibility of using these deposited fragments of CK as stable targets for radioimmuno-localization and radioimmuno-therapy of some cancers is being investigated [77]. Another consequence of the increased proteolytic activity in tumor cells is the appearance CK fragments in the sera of cancer patients. The half-life of the CK fragments in circulation is about 10-15 h, depending upon the size of the fragment [10]. The process that cause the release of soluble CK fragments into the circulation have not been completely elucidated but appear to involve multiple pathways including proteolytic degradation of CK in dying cells, abnormal mitosis, spillover of monomeric CK polypeptides from proliferating cells, apoptosis, etc. [9, 10]. Barak et al. [10] have reported that CK fragments can be detected in a number of body fluids including blood, urine, cystic fluid, ascites, pleural effusions, and CSF after their release from tumor cells. Also reported that, in normal, apparently healthy individuals, the level of CK in the circulation is low and it rises significantly in patients with carcinomas.

Squamous cell carcinomas of different sites of origin are generally characterized by a predominance of stratified-epithelial/keratinocyte-type keratins but may co-express certain simple-epithelial keratins. Most of these tumors strongly express the keratins K5, K14 and K17 normally found in the basal layer as well as the keratins K6 and



K16 characteristic for hyperproliferative keratinocytes. Focally, there may be expression of K1/K10 (particularly in higher differentiated tumor cells which can end in the formation of horn pearls), and—to a lesser extent—K4 and K13. The co-expression of simple epithelia-typical keratins comprises K8, K18, and K19, and different studies have suggested that this co-expression seems to be more pronounced in poorly differentiated squamous cell carcinoma [78]. Recently it has been demonstrated that in squamous cell carcinomas of the oral cavity the expression of K8 and K18 is an independent prognostic marker and indicates a decreased overall and progression-free survival [76]. Stratified-epithelial keratins, in particular K5 and K6, are useful as general markers for squamous cell carcinomas in histologically uncertain, poorly differentiated, or metastatic tumor cases. Although certain differences between the keratin expression patterns of squamous cell carcinomas from different sites of origin have been noted, it is not yet possible to use keratins as specific site markers in cases of unclear metastases [78].

#### **5.2.1.1 Serum CK fragments as tumor markers**

Cytokeratin fragments in serum, offer a simple, non-invasive, cheap, and reliable tool for more efficient management of cancer. As described earlier, TPA, TPS, and Cyfra 21-1 are being mainly used as prognostic markers [10]. The levels of these CK fragments in serum can be quantified using various commercially available specific serological assays.

The clinical value of determining soluble CK protein fragments in body fluids lies in the early detection of recurrence and the fast assessment of the efficacy of response to therapy in carcinomas [10]. In addition to this, other serum tumor markers have also been examined for their value in the management of various malignancies. To list a few, in HNSCC, SCCAg, CEA, [79]. In lung cancer, CEA, SCCAg, CA Ag 19.9, Neuron specific enolase (NSE), progastrin releasing peptide (ProGRP), [10]. In breast cancer, CA15-3, [80]. In gastrointestinal cancer CEA, CA-242, [81]. In cervix cancer CA 125, have been examined [10]. Only a few of these markers have proven to be clinically beneficial in any particular type of cancer. For the others diagnostic sensitivity needs to improve, especially at early stages of disease progression.

### Tissue polypeptide antigen (TPA)

This is one of the oldest tumor markers in use. It has been shown that TPA is immunologically related to a mixture of non-epidermal CK, like CK 8, 18, and 19 [82]. TPA is produced during the S and G2 phases of cell cycle. It is secreted into the circulation during and immediately after mitosis, it has been shown that the concentration of the antigen is higher in the tumor tissues and in the serum of cancer patients as compared to normal tissues or normal serum, respectively [82]. Due to its broad specificity, TPA is not being used frequently as a tumor marker in recent years.

### Tissue polypeptide specific antigen (TPS)

Tissue polypeptide specific antigen was identified long ago in human carcinomas and cell lines by the using of antibodies directed toward insoluble tumor material [83]. These antibodies have been shown to stain cytoskeletal intermediate filaments in HeLa cells [84]. It is a specific cytokeratin-based assay, which detects a defined epitope structure located on the rod domain within aminoacid (aa) residues 322-342 of human CK 18 using M3 monoclonal antibody [85]. This M3 antibody reacted with a 45-kDa protein corresponding to CK 18 on immunoblots of proteins extracted from various epithelial cell lines, while it stained three bands, 45, 33, and 29 kDa on immunoblots of proteins isolated from MCF-7 culture fluid. The same bands could be detected with CK 18-specific MAb, indicating that they represent CK 18 and its degradation products [85].

### Cyfra 21-1

This marker is recognized by two monoclonal antibodies against fragments of CK 19 in the serum. CK 19 is a type –I CK which is released into the serum as soluble fragments. CK 19 is a 40 protein sequence. The epitopes of the two antibodies were determined to be within helix 2B of the rod domain of CK 19, the epitope sequences lie within the a.a sequence 311-335 for the catcher antibody Ks 19.1 and within 346-367 for the detector BM 19.21. These sequences are unique, as could be confirmed from sequence database [9]. Both these antibodies raised by immunization of mice

with MCF-7 cells [86]. The type I keratin K19 is the smallest keratin and is exceptional since it widely lacks the non- $\alpha$ -helical tail domain typical for all other keratins [87]. It may have evolved from keratinocyte keratins. As detectable by several specific and well-tested monoclonal antibodies, K19 exhibits a rather broad tissue distribution. It is expressed in most simple epithelia (excluding parenchymatous cells such as hepatocytes, pancreatic acinar cells, and renal proximal tubular cells), notably in various ductal epithelia, in small and large intestinal epithelium, in gastric foveolar epithelium, and in mesothelium. Furthermore, it is present in most cells of pseudostratified epithelia and urothelium as well as in basal cells of non-keratinizing stratified squamous epithelia [88].

Functionally, keratin K19 is dispensable since K19 knock-out mice were viable, fertile, and appeared normal. This is apparently due to functional compensation by K18, since only mice, double deficient for K18 and K19 exhibited a severe phenotype with trophoblast fragility and early embryonic lethality. No mutation of the human K19 gene causing a disease has yet been found [89].

The expression of K19 may be induced in certain epithelia that normally lack this keratin by pathological alterations. One example is damage to renal proximal tubular epithelia by various types of injury as discussed above. K19 induction is also observed in suprabasal stratified squamous epithelial cells of oral mucosa with epithelial dysplasia, but also with inflammation, so that K19 cannot be used as a specific marker for dysplasia in oral mucosa. In carcinomas, K19 is widely expressed in both adenocarcinomas and squamous cell carcinomas and therefore is not extensively used as an immunohistochemical marker for carcinoma subtyping. One example for such application may be, in liver tumors, the distinction of hepatocellular carcinomas, which show little expression of K19, from cholangiocarcinomas and adenocarcinoma metastases, which strongly stain for this keratin [90]. The detection of soluble K19 fragments in the serum released by carcinoma cells by the Cyfra 21-1 assay has found broad clinical application as a marker to monitor treatment and evaluate response to therapy and has proven particularly useful in the case of squamous cell carcinomas of the lung [10].

Variable commercial serological kits, used for the evaluation of Cyfra21-1 as a serum marker (e.g., ELISA, ECLIA, IRAM) [31, 33, 91, 92].

### **5.2.1.2 Serum CK markers in prognosis and surveillance of cancers**

#### **a) Lung Cancer**

Histological differentiation and staging of lung cancer is essential for strategic determination of therapeutic modalities. Serum tumor markers NSE, CEA, Cyfra 21-1, proGRP, SCCAg, etc. have been shown to have considerable potential for differential diagnosis and subtyping of lung cancer [93]. It has also shown that highly elevated concentrations of CEA, Cyfra 21-1, NSE, SCCAg, and proGRP are suggestive of malignancy. Over expression of some of these markers in the serum is found to be specific for each subtype of lung cancer, e.g., CEA in adenocarcinoma; Cyfra 21-1, and SCCAg in squamous cell carcinoma; Cyfra 21-1 and NSE in large cell cancer and NSE and proGRP in small cell lung cancer [93]. Among these markers Cyfra 21-1 has been shown to have greatest potential and to be an independent prognostic factor in NSCLC [94] and the marker of choice for screening and monitoring of lung cancer, post-therapeutic surveillance, and indicator of advanced disease [94, 95] .

#### **b) Breast cancer**

Overall, the literature shows that TPA and TPS either singly or in combination with CA 15-3 are better markers for monitoring treatment during the progression of breast cancer [96].

#### **c) Ovarian cancer**

More recent clinical information derived from various studies shows that combined use of CA 125 and CK markers has additional prognostic value during monitoring of ovarian cancer patients [10]. Combination of CA-125 and TPS has been shown to significantly improve the assessment of response to treatment and consequently better outcome of the patients with ovarian cancer [97]. Cyfra 21-1 levels have been

shown to predict response to chemotherapy and for follow-up after surgery and chemotherapy [98].

#### d) Esophageal cancer

Several serum tumor markers studied, Cyfra 21-1 is not only a reliable marker for the early predilection of disease progression in esophageal SCC but it is also an invaluable tool for monitoring the efficacy of therapy and follow up [99].

### **5.3 The role of Cyfra 21-1 as a serum tumor marker in head and neck cancer**

Few studies have shown Cyfra 21-1 a highly sensitive and specific marker, providing a valuable prognostic indicator for the detection of recurrent disease and also for the evaluation of response to treatment [100]. However, their use in early diagnosis of disease is restricted because increase in CK levels in sera of HNSCC is based on tumor burden rather than the stage of the disease [100]. Some other investigators have evaluated the potential of Cyfra 21-1, TPS, SCCAg as markers for monitoring patient response to therapy and to detect early relapse in head and neck cancer [79].

Doweck et al [33] reported that Cyfra 21-1 can be used in SCCHN region at a sensitivity of 60%, with a good correlation with tumor stage and an inverse correlation with the grade of tumor differentiation. Their further studies showed that measurements of Cyfra 21-1 levels in blood provides a simple, non-invasive test to the head and neck oncologist as a prognostic tool and an additional monitoring system for early recognition of progression of the disease.

Niemann et al. [101] have demonstrated a clear correlation between tumor growth, lymph node metastases and Cyfra 21-1 serum levels. Cyfra 21-1 was found to be a helpful serological marker in the follow up of patients and it has also been proposed as a marker for monitoring head and neck cancers. According to Deng et al. [91] serum Cyfra 21-1 may be appropriate for clinical use as a reliable tumor marker for HNSCC. Mass et al. [32] are the first workers to show a potential role of Cyfra 21-1 as a serological marker for the detection of distant metastases (sites- pulmonary,

liver, osseous, cutaneous, mediastinal), or local and neck recurrences in HNSCC. Although, Hoffmann-Fazel et al. [92] have observed low sensitivity of Cyfra 21-1 for detection of primary tumor, they found it to be a good screening marker for distant metastases (sites – lung, liver, brain, skin, mediastinum), second primary tumor, and loco-regional recurrence of the tumor, respectively, in head and neck cancer. In contrast, Pradier et al. [102] did not find Cyfra 21-1 to be an appropriate parameter in identifying patients with head and neck cancer at risk of either residual disease after treatment, or recurrent or progressive disease.

Wollenberg et al. [31] also did not find any superiority for Cyfra 21-1 as compared to SCCAg and CEA with regard to their sensitivity at the time of first diagnosis of relapse. Previous studies have shown that Cyfra 21-1 levels drop to below cut-off levels 24 h after successful surgery. On the other hand, Doweck et al. [33] reported that Cyfra 21-1 had a mean lead-time of 4.1 months, in which the increased marker levels predicted the clinical detection of the recurrent disease. Thus, there appears to be some contradiction between these two observations and the mechanism of release of CK fragments into the circulation is still unclear [79]. Some investigators detected elevated Cyfra 21-1 levels in patients who were treated with radiotherapy, both during treatment and 2-3 months after completing the treatment. Detection of high levels of Cyfra 21-1 during this period is possibly due to the effect of continuous cell damage and tumor necrosis due to radiation rather than recurrence of disease [33]. Deng et al. [91] have reported that head and neck cancer patients who show local recurrence or distant metastasis within 1-6 months after surgery remain Cyfra 21-1 positive even after treatment.

Review of the literature for studies reporting the status of this marker in serum of oral SCC patients. Nagler et al. [103] reported high rates of sensitivity and specificity for Cyfra 21-1 (84% and 93%, respectively) and TPS (69% and 87% respectively). They found elevated Cyfra 21-1 levels during follow-up correlating with the detection of recurrence and second primaries. Further they have shown significant reduction in the level of each CK in the serum of oral cancer patients with approximately 2-3 weeks after resection of the tumor. Simple epithelial specific CK 8, 18, and 19 are normally not expressed in oral tissues, however, they are aberrantly expressed in oral SCC [74, 75]. Recently, expression of CK 19 in oral SCC has been linked with poor prognosis of patients [76].

From the above studies, it is apparent that there is contradictory information emerging from different laboratories, this may be because the head and neck region has been evaluated as a whole without consideration for sub-sites. Also, the information about antibodies from the commercial kits available for these markers has shown that these antibodies have developed against two-three specific epitopes in the respective CK. It is possible that these antibodies show low sensitivity in cancers other than lung cancer because there are extremely low levels of these fragments or the CK fragments released are different from those seen in lung cancer and antibodies available are unable to detect them due to conformation specificity [91]. The controversy about the usefulness of Cyfra 21-1 as serum tumor marker in head and neck squamous cell carcinoma is probably due to difficulties to find the appropriate cut-off level [12]. Cyfra 21-1 serum levels in patients with head and neck cancer are generally lower than in patients with lung cancer and they are often even equivalent to levels which are considered normal in lung cancer patients. Cytokeratins are not organ specific, and they appear in all epithelial tumors, as well as in normal epithelium. This is a limitation on the tumor marker potential of Cyfra 21-1 [13, 14].

Recent expression profiling studies show that each sub-site in the head and neck region has an unique molecular signature, thereby necessitating the evaluation of the levels of CK fragments in serum for each of these sites individually with larger number of patients [79, 104].

In this evaluation this concept was applied, so the serum level of Cyfra 21-1 was evaluated in two major sub-sites of the head and neck, laryngeal and hypopharyngeal tumors, as they share many similarities with a good number of patients (n=50), without determination of certain cut-off level for thresholds between normal and pathological values.

In this evaluation, 64% of our patients had complete remission, 36% had local residual disease and/or distant metastasis. The most common site for distant metastasis was the lung (63%), but it was obvious that any organ can be affected by distant metastasis (liver, bone, skin, suprarenal gland, etc.).

The prognostic potential of Cyfra 21-1 in the present study was evaluated by detecting the abrupt rise over time in the serum level of Cyfra 21-1 in individual

patient during follow-up, so we collect the serum levels for each individual patient at the time of primary diagnosis, 6-8 weeks post therapy (either surgery or chemo-radiotherapy or combined), and at least one reading on follow-up in a period extending from 6 months-1.5 year (available data). In this retrospective study, we have one limitation, that not all patients had serial measurements for serum Cyfra 21-1 on follow-up, as it is more informative to have serial readings on short intervals during the period of follow-up to pick up early the abrupt rise in serum levels and preferable to draw that graphically .

In the present study, no significant correlation exist between the serum concentration of Cyfra 21-1 at time of initial diagnosis, with the clinicopathological parameters [primary tumor ( $p=0.916$ ), N-status ( $p=0.424$ ), and histological grade ( $p=0.462$ )], nor with the clinical stage ( $p=0.504$ ).

Analysis of serum Cyfra 21-1 level for the first group of patients (complete remission) shows absence of abrupt rise in serum Cyfra 21-1 level on serial measurements during follow-up which was competent and correlated with clinical course of the disease in those patients.

Analysis of serum Cyfra 21-1 level for the second group of patients (recurrence and distant metastases) shows abrupt rise in serum level of Cyfra 21-1 during follow-up in (11/18) patients with clinical diagnosis of local residual disease, recurrence and / or distant metastasis.

The clinical performance of Cyfra 21-1 as a tumor marker for follow-up of our patients for a range of decision levels to separate those patients with local residual disease and distant metastasis from those patients without this condition was good. This is shown by the AUC (0.873) of their ROC curves and the sensitivity and specificity of Cyfra 21-1 at a cut-off 3.3 were 61.1% and 96.9% respectively, and also shown by the increase in the median value of Cyfra 21-1 (2 folds) at time of follow-up with progressive increase of the inter-quartile range (IQR=2.9 folds) for those patients with local recurrence and/or distant metastases.

However, the sensitivity and specificity of a test are dependent on the cut-off value that is used. Doweck et al. [100] reported that at a cut-off 1.3ng/ml, the sensitivity of Cyfra 21-1 as a tumor marker for squamous cell carcinoma of head and neck was



60%, and the specificity was 94%. Niemann et al. [12] determined the cut-off level of Cyfra 21-1 for patients with SCCHN to be 2.2ng/ml. Many other authors suggest even a lower cut-off level reaching up to 1ng/ml [105].

The ideal cut-off level for patients with HNSCC is still a matter of controversy, as when you select a higher cut-off level, the false positive fraction will decrease with increased specificity but on the other hand the true positive fraction and sensitivity will decrease, and when you select a lower cut-off value, then the true positive fraction and the sensitivity will increase. On the other hand, the true positive fraction will also increase, and therefore, the true negative fraction and specificity will decrease. To avoid this matter of controversy, we try in this evaluation to look for the rising level of Cyfra 21-1 during follow-up of patients rather than to concentrate on absolute values only.

## 6. Summary

Improvement in survival for head and neck cancer relies partly on the ability to predict the risk of recurrence after initial treatment. Furthermore, early detection of tumor progression provides more options for therapy and survival. Cytokeratin fraction 21-1(Cyfra 21-1) is a well accepted tumor marker with high sensitivity and specificity in non-small-cell lung cancer, especially squamous cell carcinoma (independent prognostic factor). In SCCHN, the clinical value of Cyfra 21-1 as a tumor marker has been debated inconclusively, probably due to difficulties in finding the appropriate cut-off level.

The aim of this study was to evaluate the importance of Cyfra 21-1 at the time of initial diagnosis and its potential as a tumor marker for follow up of patients with squamous cell carcinoma in two major sub-sites of the head and neck (laryngeal and hypopharyngeal tumors), repeated testing of Cyfra 21-1 during management in the individual patient and to compare Cyfra 21-1 levels at the time of initial diagnosis with subsequent levels (post-therapy, follow-up) to detect the changes in the serum levels.

A total of 50 patients with primary diagnosis of laryngeal and hypopharyngeal SCC between 2003-2007 in the Dept. of Otolaryngology, Head and Neck Surgery, University of Marburg, Germany, were included in this evaluation. The diagnosis was confirmed by histological biopsy findings. Tumor extent, nodal involvement, and distant metastases were assessed by a detailed physical examination, endoscopic examination and imaging investigations (B-mode ultrasonography of the neck, chest CT, neck CT, bone scan, liver scan, etc). All patients were staged according to the International Union against Cancer (UICC), TNM classification system.

Cyfra 21-1 serum levels of 50 patients with laryngeal and hypopharyngeal SCC were evaluated by ECLIA assay [CK -19 Two MAbs Ks 19.1(aa 311-335) and BM 19.21 (aa 346-367) located within helix 2B of the rod domain]. These patients had serial measurements of Cyfra 21-1 during their clinical course, so the serum level of Cyfra 21-1 was evaluated at three times [primary diagnosis, post-therapy (6-8 weeks), follow-up (at least one reading)]. The data for clinical follow-up were available from the medical file for each patient. Cyfra 21-1 levels were correlated to the clinical course of the patients and this study was independent of any reference values, for

example, healthy individuals, because only the change over time of the Cyfra 21-1 serum level in the individual patient was correlated with the individual clinical course.

Cyfra 21-1 serum concentration is not a suitable tumor marker for early diagnosis of squamous cell carcinoma of the larynx and hypopharynx as a wide range of serum Cyfra 21-1 level at time of diagnosis obtained from 0.32-13ng/ml, a mean=1.95ng/ml, a median=1.4ng/ml, and no significant correlation exist between its serum level at the time of initial diagnosis and the clinicopathological parameters [T-state ( $p=0.916$ ), N-state ( $p=0.424$ ), Histological grade ( $p=0.462$ )], nor with the clinical stage of the tumor ( $p=0.504$ ).

The clinical performance of Cyfra 21-1 as a tumor marker for follow-up of our patients for a range of decision levels to separate those patients with local residual disease and distant metastasis from those patients without this condition was good. This is shown by the AUC (0.873) of their ROC curves, and the sensitivity and specificity for Cyfra 21-1 as tumor marker for follow-up of patients with laryngeal and hypopharyngeal SCC at cut-off 3.3 were 61.1% and 96.9% respectively and also shown by the increase in the median value of Cyfra 21-1 (2 folds) at time of follow-up with progressive increase of the inter-quartile range (IQR=2.9 folds) for those patients with local residual disease and metastases.

An abrupt increase of Cyfra 21-1 in serial measurements during follow-up, indicate impending disease progression and provide early prognostic information – particularly on tumor progression and metastatic formation in the individual patient, therefore, Cyfra 21-1 serum concentration is a good marker for follow-up in patients with squamous cell carcinoma of larynx and hypopharynx to detect residual or recurrent disease and distant metastases early and in the case of abrupt rise of Cyfra 21-1 serum concentration, staging procedures are recommended.

Finally, it is important to note that the present study is retrospective. Further prospective trials are encouraged to investigate the time of the rising level of this marker in relation to the course of disease progression and to possible establish its routine use in clinical practice.

## **Zusammenfassung**

Die Verbesserung der Überlebensrate für Karzinome im Kopf-Hals-Bereich hängt zu einem nicht unerheblichen Teil davon ab, wie genau und zu welchem Zeitpunkt die Rezidivwahrscheinlichkeit nach initialer Behandlung vorausgesagt werden kann. In diesem Zusammenhang spielen sog. Tumormarker für verschiedene Tumorentitäten eine relevante Rolle. Ein derartiger Tumormarker ist das Cyfra 21-1 mit hoher Sensitivität und Spezifität bei nicht-kleinzelligen Lungenkarzinomen, insbesondere Plattenepithelkarzinomen (als unabhängiger Prognosefaktor). Vor diesem Hintergrund stellt sich unverändert die Frage, inwieweit Cyfra 21-1 auch für Plattenepithelkarzinome des Kopf-Hals-Bereiches als Tumormarker in Frage kommt.

Ziel der vorliegenden Untersuchung war die Analyse des Tumormarkerpotentials von Cyfra 21-1 bei Patienten mit Plattenepithelkarzinomen des Kehlkopfes und des Hypopharynx im Follow-up der Erkrankung. Der Cyfra 21-1 Wert wurde während des Beobachtungszeitraums bei jedem Patienten erhoben und mit den Cyfra 21-1 Werten zum Zeitpunkt der Diagnose, nach der Therapie und während des Follow-up verglichen, um Veränderungen der jeweiligen Serumwerte festzustellen.

Es wurden insgesamt 50 Patienten mit Plattenepithelkarzinomen des Kehlkopfes bzw. des Hypopharynx in die Auswertung einbezogen, die zwischen 2003 und 2007 an der Marburger Univ.-HNO-Klinik diagnostiziert wurden. Die Diagnose war in jedem Fall histologisch gesichert. Tumorausmaß, Lymphknotenstatus und Fernmetastasen wurden durch entsprechende Staging-Verfahren definiert. Das Staging erfolgte gemäß den Richtlinien der UICC (International Union against Cancer) und der TNM-Klassifikation.

Die Cyfra 21-1 Serumlevel wurden mittels ECLIA [CK-19 und BM 19.21] ermittelt. Für jeden Patienten wurde der Cyfra-Wert wenigstens dreimal bestimmt [Primärdiagnose, nach Therapie (6-8 Wochen), sowie im Verlauf der Nachsorge]. Die Daten für das klinische Follow-up wurden aus den Patientenakten erhoben.

Die Ergebnisse zusammenfassend konnte festgestellt werden, dass Cyfra 21-1 kein geeigneter Tumormarker bei der Frühdiagnose von Plattenepithelkarzinomen des Larynx oder Hypopharynx ist. Es gibt keine Korrelation zwischen dem Serumlevel zum Zeitpunkt der Erstdiagnose und den klinisch-pathologischen Parametern [T-

Klassifikation ( $p=0,916$ ), N-Klassifikation ( $p=0,424$ ), histologisches Grading ( $p=0,462$ ) sowie dem klinischen Stadium des Tumors ( $p=0,504$ ).

Die klinische Bedeutung des Cyfra 21-1 Wertes als Tumormarker im Rahmen des Follow-up der untersuchten Patienten zeigte überwiegend gute Korrelationen hinsichtlich der Unterscheidung zwischen Patienten mit lokalen Residuen und Fernmetastasen. Dies ließ sich in Form von ROC-Kurven erkennen. Die Sensitivität und Spezifität des Cyfra 21-1 als Tumormarker beim Follow-up von Patienten mit Plattenepithelkarzinomen des Kehlkopfes oder des Hypopharynx lag bei einem Cut-off Wert von 3,3 bei 61,1% bzw. 96,9%. Hinzu kam ein Anstieg des medianen Cyfra 21-1 Wertes im Rahmen des Follow-up mit progressiver Zunahme der Interquartile Range (IQR=2,9-fach) bei Patienten mit lokalen Residuen und Metastasen.

Ein abrupter Anstieg des Cyfra 21-1 Wertes während des Follow-up zeigt in aller Regel eine Progression der Erkrankungen auf und liefert frühzeitig prognostische Informationen, insbesondere was die Tumorprogression und die Metastasenbildung betrifft. Aus diesem Grund kann die Cyfra 21-1 Konzentration als relevanter Marker im Rahmen des Follow-up von Patienten mit Plattenepithelkarzinomen des Kehlkopfes und Hypopharynx betrachtet werden, vor allem dann, wenn es darum geht, Residuen, Rezidive und Fernmetastasen frühzeitig zu erkennen bzw. zu differenzieren. Im Falle eines steilen Anstiegs der Cyfra 21-1 Serumkonzentration sind weitere Staging-Verfahren empfohlen.

Die vorliegende Untersuchung wurde retrospektiv durchgeführt. Prospektive Untersuchungen sind vonnöten, um den Zeitpunkt des Konzentrationsanstiegs des Cyfra 21-1 im Vergleich zum Krankheitsverlauf beurteilen und diesen Marker verstärkt in der klinischen Praxis einsetzen zu können.

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## **Ehrenwörtliche Erklärung**

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel „Cyfra 21-1 as a Serum Tumor Marker for Follow-up of Patients with Laryngeal and Hypopharyngeal Squamous Cell Carcinoma“ im Zentrum für Hals- Nasen- und Ohrenheilkunde unter Leitung von Prof. Dr. J. A. Werner mit Unterstützung von Prof. Dr. J. A. Werner ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe.

Ich habe bisher an keinem in- oder ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

Die Veröffentlichung der Arbeit ist vorgesehen.

Marburg, den

Hani Al-Shagahin